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(57) Abstract

Multimeric hybrid genes encoding the corresponding chimeric protein comprise a gene sequence coding for an antigenic region of a protein from a first pathogen linked to a gene sequence coding for an antigenic region of a protein from a second pathogen. The pathogens particularly are parainfluenza virus (PIV) and respiratory syncytial virus (RSV). A single recombinant immunogen is capable of protecting infants and similar susceptible individuals against diseases caused by both PIV and RSV.

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CHIMERIC IMMUNOGENS

FIELD OF INVENTION

The present invention relates to the engineering and expression of multimeric hybrid genes containing sequences from the gene coding for immunogenic proteins or protein fragments of numerous pathogens.

BACKGROUND TO THE INVENTION

The advantage of the approach taken by the present invention is to produce single immunogens containing protective antigens from a range of pathogens. Such chimeras greatly simplify the development of combination vaccines, in particular, with the view ultimately to produce single dose multivalent vaccines. Multivalent vaccines are currently made by separately producing pathogens and/or their pertinent antigens and combining them in various formulations. This is a labour intensive, costly and complex manufacturing procedure. In contrast, the availability of a single immunogen capable of protecting against a range of diseases would solve many of the problems of multivalent vaccine production. Several chimeric immunogens of the type provided herein may be combined to decrease the number of individual antigens required in a multivalent vaccine.

Human Parainfluenza virus types 1,2,3 and Respiratory syncytial virus types A and B are the major viral pathogens responsible for causing severe respiratory tract infections in infants and young children. It is estimated that, in the United States alone, approximately 1.6 million infants under one year of age will have a clinically significant RSV infection each year and an additional 1.4 million infants will be infected with PIV-3. Approximately 4000 infants less than one year of age in the United States die each year from complications arising from severe respiratory tract disease caused by infection with RSV and PIV-3. The WHO

and NIALD vaccine advisory committees ranked RSV number two behind HIV for vaccine development while the preparation of an efficacious PIV-3 vaccine is ranked in the top ten vaccines considered a priority for vaccine development.

Safe and effective vaccines for protecting infants against these viral infections are not available and are urgently required. Clinical trials have shown that formaldehyde-inactivated and live-attenuated viral vaccines failed to adequately protect vaccinees against these infections. In fact, infants who received the formalin-inactivated RSV vaccine developed more serious lower respiratory tract disease during subsequent natural RSV infection than did the control group. [Am. J. Epidemiology <u>89</u>, 1969, p.405-421; J. Inf. Dis. <u>145</u>, 1982, p.311-319]. Furthermore, RSV glycoproteins purified by immunoaffinity chromatography using elution at acid pH induced immunopotentiation in cotton rats. [Vaccine, 10(7), 1992, p.475-484]. The development of efficacious PIV-3 and RSV vaccines which do not cause exacerbated pulmonary disease in vaccinees following injection with wild-type virus would have significant therapeutic implications. It is anticipated that the development of a single recombinant immunogen capable of simultaneously protecting infants against diseases caused by infection with both Parainfluenza and Respiratory syncytial viruses could significantly reduce the morbidity and mortality caused by these viral infections.

It has been reported that a protective response against PIV-3 and RSV is contingent on the induction of neutralizing antibodies against the major viral surface glycoproteins. For PIV, these protective immunogens are the HN protein which has a molecular weight of 72 kDa and possesses both hemagglutination and neuraminidase activities and the fusion (F) protein, which has a molecular weight of 65 kDa and which is responsible for

both fusion of the virus to the host cell membrane and cell-to-cell spread of the virus. For RSV, the two major immunogenic proteins are the 80 to 90 kDa G glycoprotein and the 70 kDa fusion (F) protein. The G and F proteins are thought to be functionally analogous to the PIV HN and F proteins, respectively. The PIV and RSV F glycoproteins are synthesized as inactive precursors (FO) which are proteolytically cleaved into N-terminal F2 and C-terminal F1 fragments which remain linked by disulphide bonds.

Recombinant surface glycoproteins from PIV and RSV have been individually expressed in insect cells using the baculovirus system [Ray et al., (1989), Virus Research, 12: 169-180; Coelingh et al., (1987), Virology, 160: 465-472; Wathen et al., (1989), J. of Inf. Dis. 159: 253-263] as well as in mammalian cells infected with recombinant poxviruses [Spriggs, et al., (1987), J. Virol. 61: 3416-3423; Stott et al., (1987), J. Virol. 61: 3855-3861]. Recombinant antigens produced in these systems were found to protect immunized cotton rats against live virus challenge. More recently, hybrid RSV F-G [Wathan et al., (1989), J. Gen Virol. 70: 2625-2635; Wathen, published International Patent application WO 89/05823] and PIV-3 F-HN [Wathen, published International Patent Application WO 89/10405], recombinant antigens have been engineered and produced in mammalian and insect cells. The RSV F-G hybrid antigen was shown to be protective in cotton rats [Wathan et al., (1989), J. Gen. Virol. 70: 2637-2644] although it elicited a poor anti-G antibody response [Connors et al., (1992), Vaccine 10: 475-484]. The protective ability of the PIV-3 F-HN protein was not reported in the published patent application. These antigens were engineered with the aim to protect against only the homologous virus, that is either RSV or PIV-3. However, it would be advantageous and economical to engineer and produce a single

recombinant immunogen containing at least one protective antigen from each virus in order simultaneously to protect infants and young children against both PIV and RSV infections. The chimeric proteins provided herein for such purpose also may be administered to pregnant women or women of child bearing age to stimulate maternal antibodies to both PIV and RSV. In addition, the vaccine also may be administered to other susceptible individuals, such as the elderly.

SUMMARY OF INVENTION

In its broadest aspect, the present invention provides a multimeric hybrid gene, comprising a gene sequence coding for an antigenic region of a protein from a first pathogen linked to a gene sequence coding for an antigenic region of a protein from a second pathogen and to a chimeric protein encoded by such multimeric hybrid gene. Such chimeric protein comprises an antigenic region of a protein from a first pathogen linked to an antigenic region of a protein from a second pathogen.

The first and second pathogens generally are selected from bacterial and viral pathogens and, in one embodiment, may both be viral pathogens. Preferably, the first and second pathogens are selected from those causing different respiratory tract diseases, which may be upper and lower respiratory tract diseases. In a preferred embodiment, the first pathogen is parainfluenza virus and the second pathogen is respiratory syncytial virus. The PIV protein particularly is selected from PIV-3 F and HN proteins and the RSV protein particularly is selected from RSV G and F proteins. Another aspect of the invention provides cells containing the multimeric hybrid gene for expression of a chimeric protein encoded by the gene. Such cells may be bacterial cells, mammalian cells, insect cells, yeast cells or fungal cells. Further, the present invention provides a live vector for antigen delivery containing the multimeric

hybrid gene, which may be a viral vector or a bacterial vector, and a physiologically-acceptable carrier therefor. Such live vector may form the active component of a vaccine against diseases caused by multiple pathogenic infections. Such vaccine may be formulated to be administered in an injectable form, intranasally or orally.

In an additional aspect of the present invention, there is provided a process for the preparation of a chimeric protein, which comprises isolating a gene sequence coding for an antigenic region of a protein from a first pathogen; isolating a gene sequence coding for an antigenic region of a protein from a second pathogen; linking the gene sequences to form a multimeric hybrid gene; and expressing the multimeric hybrid gene in a cellular expression system. Such cellular expression system may be provided by bacterial cells, mammalian cells, insect cells, yeast cells or fungal cells. The chimeric protein product of gene expression may be separated from a culture of the cellular expression system and purified.

The present invention further includes a vaccine against diseases caused by multiple pathogen infections, comprising the chimeric protein encoded by the multimeric hybrid gene and a physiologically-acceptable carrier therefor. Such vaccine may be formulated to be administered in an injectable form, intranasally or orally.

The vaccines provided herein may be used to immunize a host against disease caused by multiple pathogenic infections, particularly those caused by a parainfluenza virus and respiratory syncytial virus, by administering an effective amount of the vaccine to the host. As noted above, for human PIV and RSV , the host may be infants and young children, pregnant women as well as those of a

child-bearing age, and other susceptible persons, such as the elderly.

The chimeric protein provided herein also may be used as a diagnostic reagent for detecting infection by a plurality of different pathogens in a host, using a suitable assaying procedure.

It will be appreciated that, while the description of the present invention which follows focuses mainly on a chimeric molecule which is effective for immunization against diseases caused by infection by PIV and RSV, nevertheless the invention provided herein broadly extends to any chimeric protein which is effected for immunization against diseases caused by a plurality of pathogens, comprising an antigen from each of the pathogens linked in a single molecule, as well as to genes coding for such chimeric molecules.

In this application, by the term "multimeric hybrid genes" we mean genes encoding antigenic regions of proteins from different pathogens and by the term "chimeric proteins" we mean immunogens containing antigenic regions from proteins from different pathogens.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 shows the nucleotide (SEQ ID No: 1) and amino acid (SEQ ID No: 2) sequence of a PCR-amplified PIV-3 F gene and F protein, respectively;

Figure 2 shows the restriction map of the PIV-3 F gene;

Figure 3 shows the nucleotide (SEQ ID No: 3) and amino acid (SEQ ID No: 4) sequences of the PIV-3 HN gene and HN protein, respectively;

Figure 4 shows the restriction map of the PIV-3 HN gene;

Figure 5 shows the nucleotide (SEQ ID No: 5) and amino acid (SEQ ID No: 6) sequences of the RSV F gene and RSV F protein, respectively;

Figure 6 shows the restriction map of the RSV F gene;

Figure 7 shows the nucleotide (SEQ ID No: 7) and amino acid (SEQ ID No: 8) sequences of the RSV G gene and RSV G protein, respectively;

Figure 8 shows the restriction map of the RSV G gene;

Figure 9 shows the steps involved in the construction of an expression vector containing a chimeric F_{PIV-3} - F_{RSV} gene;

Figure 10 shows the steps involved in the construction of an expression vector containing a F_{PIV-3} gene lacking the 5'-untranslated sequence and transmembrane anchor and cytoplasmic tail coding regions;

Figure 11 shows the steps involved in the construction of an expression vector containing a chimeric F_{PIV-3} - F_{RSV} gene containing a truncated PIV-3 F gene devoid of 5'-untranslated region linked to a truncated RSV F1 gene;

Figure 12 shows the steps involved in construction of a modified pAC 610 baculovirus expression vector containing a chimeric F_{PIV-3} - F_{RSV} gene consisting of the PIV-3 F gene lacking both the 5'-untranslated sequence as well as transmembrane and cytoplasmic tail coding region linked to the truncated RSV F1 gene;

Figure 13 shows immunoblots of cell lysates from Sf9 cells infected with recombinant baculoviruses;

Figure 14 shows the steps involved in constructing a baculovirus transfer vector (pD2);

Figure 15 shows the steps involved in construction of a chimeric F_{RSV} - HN_{PIV-3} gene;

Figure 16 shows an SDS-PAGE gel and immunoblot of purified F_{RSV} - HN_{PIV-3} chimeric protein;

Figure 17 illustrates mutagenesis of a PIV-3 F gene; and

Figure 18 shows the steps involved in the construction of a chimeric F_{PIV-3} - G_{RSV} gene.

GENERAL DESCRIPTION OF INVENTION

In the present invention, a chimeric molecule protective against two different major childhood diseases is provided. The present invention specifically relates to the formulation of various recombinant Parainfluenza virus (PIV)/Respiratory syncytial virus (RSV) immunogens to produce safe and efficacious vaccines capable of protecting infants and young children, as well as other susceptible individuals, against diseases caused by infection with both PIV and RSV. However, as described above, the present invention extends to the construction of multimeric hybrid genes containing genes coding for protective antigens from many pathogens. Such vaccines may be administered in any desired manner, such as a readily-injectable vaccine, intranasally or orally.

In the present invention, the inventors have specifically engineered several model PIV/RSV chimeric genes containing relevant sequences from selected genes coding for PIV-3 and RSV surface glycoproteins linked in tandem. All genes in the chimeric constructs described herein were obtained from recent clinical isolates of PIV-3 and RSV. The chimeric gene constructs may include gene sequences from either PIV-3 F or HN genes linked in tandem to either RSV F or G genes in all possible relative orientations and combinations.

The chimeric gene constructs provided herein may consist of either the entire gene sequences or gene segments coding for immunogenic and protective epitopes thereof. The natural nucleotide sequence of these genes may be modified by mutation while retaining antigenicity and such modifications may include the removal of putative pre-transcriptional terminators to optimize their expression in eukaryotic cells. The genes were

designed to code for hybrid PIV-RSV surface glycoproteins linked in tandem in a single construct to produce gene products which elicit protective antibodies against both parainfluenza and respiratory syncytial viruses. Such multimeric hybrid genes consist of a gene sequence coding for a human PIV-3 F or HN protein or an immunogenic epitope-containing fragment thereof linked to a gene sequence coding for a human RSV G or F protein or an immunogenic epitope-containing fragment thereof. Specific gene constructs which may be employed include F_{PIV-3} - F_{RSV}, F_{RSV} - HN_{PIV-3} and F_{PIV-3} - G_{RSV} hybrid genes.

In addition, the present invention also extends to the construction of other multimeric genes, such as trimeric genes containing PIV and RSV genes or gene segments, linked in all possible relative orientations. For example:

F_{PIV} - HN_{PIV} - F or G_{RSV}

F_{PIV} - F_{RSV} - G_{RSV}

HN_{PIV} -F_{RSV} - G_{RSV}

The multimeric genes provided herein also may comprise at least one gene encoding at least one immunogenic and/or immunostimulating molecule.

The multimeric hybrid genes provided herein may be sub-cloned into appropriate vectors for expression in cellular expression systems. Such cellular expression systems may include bacterial, mammalian, insect and fungal, such as yeast, cells.

The chimeric proteins provided herein also may be presented to the immune system by the use of a live vector, including live viral vectors, such as recombinant poxviruses, adenoviruses, retroviruses, Semliki Forest viruses, and live bacterial vectors, such as Salmonella and mycobacteria (e.g. BCG).

Chimeric proteins, such as a PIV/RSV chimera, present in either the supernatants or cell lysates of

transfected, transformed or infected cells then can be purified in any convenient manner.

To evaluate the immunogenicity and protective ability of the chimeric proteins, suitable experimental animals are immunized with either varying doses of the purified chimeric proteins, such as the PIV/RSV chimera, and/or live recombinant vectors as described above. Such chimeric proteins may be presented to the immune system by either the use of physiologically-acceptable vehicles, such as aluminum phosphate, or by the use of delivery systems, such as ISCOMS and liposomes. The chimeras also may be formulated to be capable of eliciting a mucosal response, for example, by conjugation or association with immunotargeting vehicles, such as the cholera toxin B subunit, or by incorporation into microparticles. The vaccines may further comprise means for delivering the multimeric protein specifically to cells of the immune system, such as toxin molecules or antibodies. To further enhance the immunoprotective ability of the chimeric proteins, they may be supplemented with other immunogenic and/or immunostimulating molecules. The chimeric PIV/RSV proteins specifically described herein may be formulated with an adjuvant, such as aluminum phosphate, to produce readily-injectable vaccines for protection against the diseases caused by both PIV-3 and RSV. The chimeric proteins also may be administered intranasally or orally. The chimeric proteins may be used in test kits for diagnosis of infection by PIV-3 and RSV.

The invention is not limited to the preparation of chimeric PIV-3 and RSV proteins, but is applicable to the production of chimeric immunogens composed of either the entire sequences or regions of the immunogenic proteins from at least two pathogens sequentially linked in a single molecule. Chimeric antigens also may be synthesized to contain the immunodominant epitopes of

several proteins from different pathogens. These chimeric antigens may be useful as vaccines or as diagnostic reagents.

SEQUENCE IDENTIFICATION

Several nucleotide and amino acid sequences are referred to in the disclosure of this application. The following table identifies the sequences and the location of the sequence:

SEQ ID No.	Identification	Location
1	Nucleotide sequence for PCR-amplified PIV-3 F gene	Fig. 1, Example 1
2	Amino acid sequence for PCR-amplified PIV-F protein	Fig. 1, Example 1
3	Nucleotide sequence for PIV-3 HN gene	Fig. 3, Example 1
4	Amino acid sequence for PIV-3 HN protein	Fig. 3, Example 1
5	Nucleotide sequence for RSV F gene	Fig. 5, Example 1
6	Amino acid sequence for RSV F protein	Fig. 5, Example 1
7	Nucleotide sequence for RSV G gene	Fig. 7, Example 1
8	Amino acid sequence for RSV G protein	Fig. 7, Example 1
9	BsrI - BamHI oligo-nucleotide cassette	Fig. 9, Example 2
10	BspHI - BamHI oligo-nucleotide cassette	Fig. 9, Example 2
11	EcoRI - Ppu MI oligo-nucleotide cassette	Fig. 9, Example 2
12	BrsI - BamHI oligo-nucleotide cassette	Fig. 10, Example 3

13	EcoRI -Bsr BI oligo-nucleotide cassette	Fig. 10, Example 3
14	EcoRV - EcoRI oligo-nucleotide cassette	Fig. 11, Example 5
15	EcoRV - BamHI oligo-nucleotide cassette	Fig. 14, Example 8
16	BspHI - BspHI oligo-nucleotide cassette	Fig. 15, Example 9
17	Nucleotide sequence for PIV-3 F gene	Example 15
18	Mutagenic oligo-nucleotide #2721	Fig. 17, Example 15
19	Nucleotide sequence for part of oligo-nucleotide #2721	Example 15
20	Oligonucleotide probe	Example 15

DEPOSIT INFORMATION

Certain plasmid DNAs described and referred to herein have been deposited with the American Type Culture Collection (ATCC) located at Rockville, Maryland, USA, pursuant to the Budapest Treaty and prior to the filing of this application. The deposited purified plasmids will become available to the public upon grant of this U.S. patent application or upon publication of its corresponding European patent application, whichever first occurs. The invention described and claimed herein is not to be limited in scope by the plasmid DNAs of the constructs deposited, since the deposited embodiment is intended only as an illustration of the invention. The following purified plasmids were deposited at the ATCC with the noted accession numbers on December 17, 1992:

Plasmid	Example No.	Accession No.
pAC DR7	5	75387
pD2RF-HN	9	75388
pD2F-G	16	75389

Any equivalent plasmids that can be used to produce equivalent antigens as described in this application are within the scope of the invention.

EXAMPLES

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific Examples. These Examples are described solely for purposes of illustration and are not intended to limit the scope of the invention. Changes in form and substitution of equivalents are contemplated as circumstances may suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitations.

Methods for cloning and sequencing the PIV-3 and RSV genes as well as the procedures for sub-cloning the genes into appropriate vectors and expressing the gene constructs in mammalian and insect cells are not explicitly described in this disclosure but are well within the scope of those skilled in the art.

Example 1:

This Example outlines the strategy used to clone and sequence the PIV-3 F, HN and RSV F, G genes (from a type A isolate). These genes were used in the construction of the F_{PIV-3} - F_{RSV}, F_{RSV}-HN_{PIV-3}, and F_{PIV-3}-G_{RSV} chimeric genes detailed in Examples 2 to 4, 9 and 15, respectively.

Two PIV-3 F gene clones initially were obtained by PCR amplification of cDNA derived from viral RNA extracted from a recent clinical isolate of PIV-3. Two other PIV-3 F gene clones as well as the PIV-3 HN, RSV F and RSV G genes were cloned from a cDNA library prepared from mRNA isolated from MRC-5 cells infected with clinical isolates of either PIV-3 or RSV (type A isolate). The PIV-3 F (both PCR amplified and non-PCR amplified), PIV-3 HN, RSV F and RSV G gene clones were sequenced by the dideoxynucleotide chain termination

procedure. Sequencing of both strands of the genes was performed by a combination of manual and automated sequencing.

The nucleotide (SEQ ID No: 1) and amino acid (SEQ ID No: 2) sequences of the PCR amplified PIV-3 F gene and F protein, respectively, are presented in Figure 1 and the restriction map of the gene is shown in Figure 2. Sequence analysis of the 1844 nucleotides of two PCR amplified PIV-3 F gene clones confirmed that the clones were identical. Comparison of the coding sequence of the PCR-amplified PIV-3 F gene clone with that of the published PIV-3 F gene sequence revealed a 2.6% divergence in the coding sequence between the two genes resulting in fourteen amino acid substitutions.

The nucleotide sequence of the non-PCR amplified PIV-3 F gene clone differed from the PCR amplified gene clone in the following manner: the non-PCR amplified clone had ten additional nucleotides (AGGACAAAAG) at the 5′ untranslated region of the gene and differed at four positions, 8 (T in PCR-amplified gene to C in non-PCR amplified gene) , 512 (C in PCR-amplified gene to T in non-PCR amplified gene) , 518 (G in PCR-amplified gene to A in non-PCR amplified gene) and 1376 (A in PCR-amplified gene to G in non-PCR amplified gene). These changes resulted in three changes in the amino acid sequence of the F protein encoded by the non-PCR amplified PIV-3 F gene. Serine (position 110), glycine (position 112), and aspartic acid (position 398) in the primary amino acid sequence of the F protein encoded by the PCR amplified PIV-3 F gene was changed to phenylalanine (position 110), glutamic acid (position 112) and glycine (position 398), respectively, in the primary amino acid sequence of the F protein encoded by the PCR amplified clone.

Figure 3 shows the nucleotide (SEQ ID No: 3) and amino acid (SEQ ID No: 4) sequences of the PIV-3 HN gene and protein, respectively and the restriction map of the

gene is presented in Figure 4. Analysis of the 1833 nucleotide sequence from two HN clones confirmed that the sequences were identical. A 4.4% divergence in the coding sequence of the PIV-3 HN gene was noted when the sequence was compared to the published PIV-3 HN coding sequence. This divergence resulted in seventeen amino acid substitutions in the amino acid sequence of the protein encoded by the PIV-3 HN gene.

The nucleotide (SEQ ID No: 5) and amino acid (SEQ ID No: 6) sequences of the RSV F gene and RSV F protein, respectively, are shown in Figure 5 and the restriction map of the gene is shown in Figure 6. Analysis of the 1887 nucleotide sequence from two RSV F clones verified complete sequence homology between the two clones. Comparison of this nucleotide sequence with that reported for the RSV F gene revealed approximately 1.8% divergence in the coding sequence resulting in eleven amino acid substitutions.

The nucleotide (SEQ ID No: 7) and amino acid (SEQ ID No: 8) sequences of the RSV G gene and RSV G protein, respectively, are presented in Figure 7 while the restriction map of the gene is outlined in Figure 8. Comparison of the 920 nucleotide sequence of the G gene clone with the published G sequence (type A isolate) revealed a 4.2% divergence in the nucleotide sequence and a 6.7% divergence in the amino acid sequence of the gene product. This divergence resulted in twenty amino acid substitutions.

The full-length PIV-3 F (non-PCR amplified) , PIV-3 HN, RSV F and RSV G genes were cloned into λgt11 and subcloned into the multiple cloning site of a Bluescript M13-SK vector, either by blunt end ligation or using appropriate linkers. The PCR-amplified PIV-3 F gene was directly cloned into the Bluescript vector. The cloning vectors containing the PIV-3 F-PCR amplified, PIV-3 F non-PCR amplified, PIV-3 HN, RSV F and RSV G genes were

named pPI3F, pPI3Fc, pPIVHN, pRSVF and pRSVG, respectively.

Example 2:

This Example illustrates the construction of a Bluescript-based expression vector (pMCR20) containing the chimeric F_{PIV-3} - F_{RSV} gene. This chimeric gene construct contains the 5′ untranslated region of the PIV-3 F gene but lacks the hydrophobic anchor and cytoplasmic tail coding regions of both the PIV-3 and RSV F genes. The steps involved in the construction of this plasmid are summarized in Figure 9.

To prepare the PIV-3 portion of the chimeric gene (Figure 9, step 1), the full length PIV-3 gene lacking the transmembrane region and cytoplasmic tail coding regions was retrieved from plasmid pPI3F by cutting the polylinker with BamHI, blunt-ending the linearized plasmid with Klenow polymerase and cutting the gene with BsrI. A BsrI-BamHI oligonucleotide cassette (SEQ ID No: 9) containing a PpuMI site and three successive translational stop codons were ligated to the truncated 1.6 Kb [BamHI]-BsrI PIV-3 F gene fragment and cloned into the EcoRV-BamHI sites of a Bluescript M13-SK expression vector containing the human methallothionen promoter and the poly A and IVS sequences of the SV40 genome (designated pMCR20), to generate plasmid pME1.

To engineer the RSV F gene component of the chimeric construct (Figure 9, step 2), the RSV F gene lacking the transmembrane region and cytoplasmic tail coding regions was retrieved from plasmid pRSVF by cutting the polylinker with EcoRI and the gene with BspHI. A synthetic BspHI-BamHI oligonucleotide cassette (SEQ ID No: 10) containing three successive translational stop codons was ligated to the 1.6 Kb truncated RSV F gene and cloned into the EcoRI-BamHI sites of the Bluescript based expression vector, pMCR20 to produce plasmid pES13A. Plasmid pES13A then was cut with EcoRI and PpuMI to

remove the leader and F2 coding sequences from the truncated RSV F gene. The leader sequence was reconstructed using an EcoRI-PpuMI oligocassette (SEQ ID No: 11) and ligated to the RSV F1 gene segment to generate plasmid pES23A.

To prepare the chimeric F_{PIV-3}-F_{RSV} gene (Figure 9, step 3) containing the 5′ untranslated region of the PIV-3 F gene linked to the truncated RSV F1 gene fragment, plasmid pME1 (containing the 1.6 Kb truncated PIV-3 F gene) first was cut with PpuMI and BamHI. The PpuMI-BamHI restricted pME1 vector was dephosphorylated with intestinal alkaline phosphatase. The 1.1 Kb RSV F1 gene fragment was retrieved from plasmid pES23A by cutting the plasmid with PpuMI and BamHI. The 1.1 Kb PpuMI-BamHI RSV F1 gene fragment was cloned into the PpuMI-BamHI sites of the dephosphorylated pME1 vector to generate plasmid pES29A. This chimeric gene construct contains the 5′ untranslated region of the PIV-3 F gene but lacks the nucleotide sequences coding for the hydrophobic anchor domains and cytoplasmic tails of both the PIV-3 and RSV F proteins.

Example 3:

This Example illustrates the construction of a Bluescript-based expression vector containing the PIV-3 F gene lacking both the 5′ untranslated and transmembrane anchor and cytoplasmic tail coding regions. The steps involved in constructing this plasmid are outlined in Figure 10.

Plasmid pPI3F containing the full length PIV-3 F gene was cut with BamHI, blunt ended with Klenow polymerase and then cut with BsrI to remove the transmembrane and cytoplasmic tail coding regions. The Bluescript-based expression vector, pMCR20, was cut with SmaI and BamHI. A synthetic BsrI-BamHI oligonucleotide cassette (SEQ ID No: 12) containing a translational stop codon was ligated with the 1. 6 Kb blunt ended-BsrI PIV-3

F gene fragment to the SmaI-BamHI restricted pMCR20 vector to produce plasmid pMpFB. The PIV-3 F gene of this construct lacked the DNA fragment coding for the transmembrane and cytoplasmic anchor domains but contained the 5′ untranslated region. To engineer a plasmid containing the PIV-3 F gene devoid of both the 5′ untranslated region and the DNA fragment coding for the hydrophobic anchor domain, plasmid pMpFB was cut with EcoRI and BstBI. An EcoRI-BstBI oligocassette (SEQ ID No: 13) containing the sequences to reconstruct the signal peptide and coding sequences removed by the EcoRI-BstBI cut was ligated to the EcoRI-BstBI restricted pMpFB vector to produce plasmid pMpFA.

Example 4:

This Example illustrates the construction of the chimeric F_{PIV-3}-F_{RSV} gene composed of the truncated PIV-3 F gene devoid of the 5′ untranslated region linked to the truncated RSV F1 gene. The steps involved in constructing this plasmid are summarized in Figure 11.

To prepare this chimeric gene construct, plasmid pES29A (Example 2) was cut with BstBI and BamHI to release the 2.5 Kb BstBI-BamHI PI3-3 F-RSV F1 chimeric gene fragment. This BstBI-BamHI fragment was isolated from a low melting point agarose gel and cloned into the BstBI-BamHI sites of the dephosphorylated vector pMpFA to produce plasmid pES60A. This construct contained the PIV-3 F gene lacking both the 5′ untranslated region and the hydrophobic anchor and cytoplasmic tail coding sequences linked to the F1 coding region of the truncated RSV F gene. This chimeric gene was subsequently subcloned into the baculovirus transfer vector (see Example 5).

Example 5:

This Example illustrates the construction of the modified pAC 610 baculovirus transfer vector containing the native polyhedrin promoter and the chimeric F_{PIV-3}-F_{RSV}

gene consisting of the PIV-3 F gene lacking both the 5' untranslated sequence and the nucleotide sequence coding for the hydrophobic anchor domain and cytoplasmic tail linked to the truncated RSV F1 gene. Construction of this plasmid is illustrated in Figure 12.

The pAC 610 baculovirus expression vector was modified to contain the native polyhedrin promoter in the following manner. Vector pAC 610 was cut with EcoRV and BamHI. The 9.4 Kb baculovirus transfer vector lacking the EcoRV-BamHI DNA sequence was isolated from a low melting point agarose gel and treated with intestinal alkaline phosphatase. In a 3-way ligation, an EcoRV-EcoRI oligonucleotide cassette (SEQ ID No: 14) containing the nucleotides required to restore the native polyhedrin promoter was ligated with the 1.6 Kb EcoRI-BamHI truncated RSV F gene fragment isolated from construct pES13A (Example 2, step 2) and the EcoRV-BamHI restricted pAC 610 phosphatased vector to generate plasmid pES47A. To prepare the pAC 610 based expression vector containing the chimeric F_{PIV-3}-F_{RSV} gene, plasmid pES47A was first cut with EcoRI and BamHI to remove the 1.6 Kb truncated RSV F gene insert. The 2.8 Kb F_{PIV-3}-F_{RSV} chimeric gene was retrieved by cutting plasmid pES60A (Example 4) with EcoRI and BamHI. The 2.8 Kb EcoRI-BamHI chimeric gene was ligated to the EcoRI-BamHI restricted pES47A vector to generate plasmid pAC DR7 (ATCC 75387).

<u>Example 6</u>

This Example outlines the preparation of plaque-purified recombinant baculoviruses containing the chimeric F_{PIV-3} - F_{RSV} gene.

Spodoptera frugiperda (Sf9) cells were co-transfected with 1.0 μg wild-type AcMNPV DNA and 2.5 μg of F_{PIV-3}-F_{RSV} plasmid DNA (plasmid pAC DR7 - Example 5). Putative recombinant baculoviruses (purified once by serial dilution) containing the F_{PIV-3} - F_{RSV} chimeric gene were identified by dot-blot hybridization. Lysates of

insect cells infected with the putative recombinant baculoviruses were probed with the ^{32}P-labelled F_{PIV-3}-F_{RSV} chimeric gene insert. Recombinant baculoviruses were plaque-purified twice before being used for expression studies. All procedures were carried out according to the protocols outlined by M.D. Summers and G.E. Smith in "A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures", Texas Agricultural Experiment Station, Bulletin 1555, 1987.

Example 7:

This Example illustrates the presence of the chimeric F_{PIV-3} -F_{RSV} protein in supernatants and cell lysates of infected Sf9 cells.

Insect cells were infected with the plaque-purified recombinant baculoviruses prepared as described in Example 6 at a m.o.i. of 8. Concentrated supernatants from cells infected with the recombinant viruses were positive in a PIV-3 F specific ELISA. In addition, when lysates from ^{35}S-methioninelabelled infected cells were subjected to SDS-polyacrylamide gel electrophoresis and gels were analyzed by autoradiography, a strong band with apparent molecular weight of approximately 90 kDa was present in lysates of cells infected with the recombinant viruses but was absent in the lysates from wild-type infected cells. The presence of the chimeric F_{PIV-3} - F_{RSV} protein in the lysates of cells infected with the recombinant baculoviruses was confirmed further by Western blot analysis using monospecific anti-PIV-3 F and anti-RSV F antisera and/or monoclonal antibodies (Mabs). Lysates from cells infected with the recombinant baculoviruses reacted with both anti-PIV-3 and anti-RSV antisera in immunoblots. As shown in the immunoblot of Figure 13, lysates from cells infected with either the RSV F or F_{PIV-3} - F_{RSV} recombinant baculoviruses reacted positively with the anti-F RSV Mab. As expected, lysates from cells infected with wild type virus did not react

with this Mab. In addition, only lysates from cells infected with the chimeric F_{PIV-3} - F_{RSV} recombinant viruses reacted with the anti-PIV-3 F_1 antiserum.

Example 8

This Example illustrates modification of the baculovirus transfer vector pVL1392 (obtained from Invitrogen), wherein the polyhedrin ATG start codon was converted to ATT and the sequence CCG was present downstream of the polyhedrin gene at positions +4,5,6. Insertion of a structural gene several base pairs downstream from the ATT codon is known to enhance translation. The steps involved in constructing this modified baculovirus transfer vector are outlined in Figure 14.

The baculovirus expression vector pVL1392 was cut with EcoRV and BamHI. The 9.5 kb restricted pVL1392 vector was ligated to an EcoRV-BamHI oligonucleotide cassette (SEQ ID No: 15) to produce the pD2 vector.

Example 9:

This Example illustrates the construction of the pD2 baculovirus expression vector containing the chimeric F_{RSV} -HN_{PIV-3} gene consisting of the truncated RSV F and PIV-3 HN genes linked in tandem. The steps involved in constructing this plasmid are summarized in Figure 15.

To engineer the F_{RSV}-HN_{PIV-3} gene, the RSV F gene lacking the nucleotide sequence coding for the transmembrane domain and cytoplasmic tail of the RSV F glycoprotein was retrieved from plasmid pRSVF (Example 1) by cutting the polylinker with EcoRI and the gene with BspHI. The PIV-3 HN gene devoid of the DNA fragment coding for the hydrophobic anchor domain was retrieved from plasmid pPIVHN (Example 1) by cutting the gene with BspHI and the polylinker with BamHI. The 1.6 Kb EcoRI-BspHI RSV F gene fragment and the 1.7 Kb BspHI-BamHI PIV-3 HN gene fragment were isolated from low melting point agarose gels. For cloning purposes, the two BspHI sites

in the Bluescript based mammalian cell expression vector, pMCR20, were mutated. Mutations were introduced in the BspHI sites of the pMCR20 by cutting the expression vector with BspHI, treating both the BspHI restricted vector and the 1. 1 Kb fragment released by the BspHI cut with Klenow polymerase and ligating the blunt-ended 1.1 Kb fragment to the blunt-ended Bluescript-based expression vector to generate plasmid pM'. Since insertion of the 1.1 Kb blunt-end fragment in the mammalian cell expression vector in the improper orientation would alter the Amp^r gene of the Bluescript-based expression vector, only colonies of HB101 cells transformed with the pM' plasmid DNA with the 1.1 Kb blunt-ended fragment in the proper orientation could survive in the presence of ampicillin. Plasmid DNA was purified from ampicillin-resistant colonies of HB101 cells transformed with plasmid PM' by equilibrium centrifugation in cesium chloride-ethidium bromide gradients. The 1.6 Kb EcoRI-BspHI RSV F and 1.7 Kb BspHI-BamHI PIV-3 HN gene fragments were directly cloned into the EcoRI-BamHI sites of vector pM' in a 3-way ligation to generate plasmid pM' RF-HN.

To restore specific coding sequences of the RSV F and PIV-3 HN genes removed by the BspHI cut, a BspHI-BspHI oligonucleotide cassette (SEQ ID No: 16) containing the pertinent RSV F and PIV-3 HN gene sequences was ligated via the BspHI site to the BspHI-restricted plasmid pM' RF-HN to produce plasmid pM RF-HN. Clones containing the BspHI-BspHI oligonucleotide cassette in the proper orientation were identified by sequence analysis of the oligonucleotide linker and its flanking regions.

To clone the chimeric F_{RSV}-HN_{PIV-3} gene into the baculovirus expression vector pD2 (Example 8), the F_{RSV}-HN_{PIV-3} truncated gene first was retrieved from plasmid pM RF-HN by cutting the plasmid with EcoRI. The 3.3 Kb F_{RSV}-

HN_{PIV-3} gene then was cloned into the EcoRI site of the baculovirus transfer vector plasmid pD2 to generate plasmid pD2 RF-HN (ATCC 75388). Proper orientation of the 3.3 Kb EcoRI F_{RSV}-HN_{PIV-3} chimeric gene insert in plasmid pD2 RF-HN was confirmed by sequence analysis.

Example 10:

This Example outlines the preparation of plaque-purified recombinant baculoviruses containing the chimeric F_{RSV}-HN_{PIV-3} gene.

Spodoptera frugiperda (Sf9) cells were co-transfected with 1 μg wild-type AcNPV DNA and 2 μg of F_{RSV}-HN_{PIV-3} plasmid DNA (plasmid pD2 RF-HN-Example 9). Putative recombinant baculoviruses (purified once by serial dilution) containing the F_{RSV}-HN_{PIV-3} chimeric gene were identified by dot-blot hybridization. Lysates of insect cells infected with the putative recombinant baculoviruses were probed with the ^{32}P-labelled RSV F or PTV-3 HN gene oligonucleotide probes. Recombinant baculoviruses were plaque-purified three times before being used for expression studies. All procedures were carried out according to the protocols outlined by Summers and Smith (Example 6).

Example 11:

This Example illustrates the presence of the chimeric F_{RSV}-HN_{PIV-3} protein in supernatants of infected Sf9 and High 5 cells.

Insect cells (Sf9 and High 5), maintained in serum free medium EX401, were infected with the plaque purified recombinant baculoviruses of Example 10 at a m.o.i. of 5 to 10 pfu/cell. Supernatants from cells infected with the recombinant baculoviruses tested positive for expressed protein in both the RSV-F and PIV-3 HN specific ELISAS. In addition, supernatants from infected cells reacted positively with both an anti-F RSV monoclonal antibody and anti-HN peptide antisera on immunoblots. A distinct band of approximately 105 kDa was present in the

immunoblots. These results confirm the secretion of the chimeric F_{RSV}-$HN_{PIV\text{-}3}$ protein into the supernatant of Sf9 and High 5 cells infected with the recombinant baculoviruses.

Example 12:

This Example illustrates the purification of the chimeric F_{RSV}-$HN_{PIV\text{-}3}$ protein from the supernatants of infected High 5 cells.

High 5 cells, maintained in serum free medium, were infected with the plaque purified recombinant baculoviruses of Example 10 at a m.o.i of 5 pfu/cell. The supernatant from virus infected cells was harvested 2 days post-infection. The soluble F_{RSV}-$HN_{PIV\text{-}3}$ chimeric protein was purified from the supernatants of infected cells by immunoaffinity chromatography using an anti-HN PIV-3 monoclonal antibody. The anti-HN monoclonal antibody was coupled to CNBr-activated Sepharose 4B by conventional techniques. The immunoaffinity column was washed with 10 bed volumes of washing buffer (10mM Tris-HCl pH 7.5, 150 mM NaCl, 0.02% v/v Triton-X 100) prior to use. After sample loading, the column was washed with 10 bed volumes of washing buffer followed by 3 bed volumes of high salt buffer (10mm Tris-HCl pH 7.5, 500mM NaCl, 0.02% v/v Triton-X 100) . The chimeric F_{RSV}-$HN_{PIV\text{-}3}$ protein was eluted from the immunoaffinity column with 100 MM glycine, pH 2.5, in the presence of 0.02% Triton X-100. Eluted protein was neutralized immediately with 1M Tris-HCl, pH 10.7.

Polyacrylamide gel electrophoretic analysis (Fig. 16, panel A) of the immunoaffinity-purified F_{RSV}-$HN_{PIV\text{-}3}$ protein revealed the presence of one major protein band with an apparent molecular weight of 105 kDa. The purified protein reacted with both an anti-RSV F monoclonal antibody and anti-HN peptide antisera on immunoblots (Fig. 16, panel B, lanes 1 and 2, respectively).

Example 13:

This Example illustrates the immunogenicity of the F_{RSV}-HN_{PIV-3} protein in guinea pigs.

Groups of four guinea pigs were injected intramuscularly with either 1.0 or 10.0 μg of the chimeric F_{RSV}-HN_{PIV-3} protein purified as described in Example 12 and adjuvanted with aluminum phosphate. Groups of control animals were immunized with either placebo, or live PIV-3 or RSV (administered intranasally). Guinea pigs were bled 2 and 4 weeks after the primary injection and boosted at 4 weeks with an equivalent dose of the antigen formulation. Serum samples also were taken 2 and 4 weeks after the booster dose. To assess the ability of the chimeric protein to elicit PIV-3 and RSV-specific antibody responses, sera samples were analyzed for the presence of PIV-3 specific hemagglutination inhibiting and neutralizing antibodies as well as RSV neutralizing antibodies. As summarized in Table 1 below (the Tables appear at the end of the disclosure), the sera of animals immunized with two 10 μg doses of the chimeric protein had titres of PIV-3 specific hemagglutination inhibition (HAI) and PIV-3/RSV neutralizing antibodies at the 6 and 8 week time points which were equivalent to the levels obtained following intranasal inoculation with either live PIV-3 or RSV. In addition, animals immunized with only two 1 ug doses of the chimeric protein elicited strong PIV-3 and RSV specific neutralizing antibodies. These results confirmed the immunogenicity of both the RSV and PIV-3 components of the chimeric protein and provided confirmatory evidence that a single recombinant immunogen can elicit neutralizing antibodies against both RSV and PIV-3.

Example 14:

This Example illustrates the immunogenicity and protective ability of the F_{RSV}-HN_{PIV-3} protein in cotton rats.

Groups of eight cotton rats were injected intramuscularly with either 1.0 or 10.0 ug of the chimeric F_{RSV}-HN_{PIV-3} protein (prepared as described in Example 12) adjuvanted with aluminum phosphate. Groups of control animals were immunized with either placebo (PBS + aluminum phosphate) or live PIV-3 or RSV (administered intranasally). Cotton rats were bled 4 weeks after the primary injection and boosted at 4 weeks with an equivalent dose of the antigen formulation. Serum samples were also taken 1 week after the booster dose. As shown in Table 2 below, data from the 4-week bleed demonstrated that both a 1 and 10 μg dose of the chimeric protein was capable of inducing a strong primary response. Reciprocal mean $\log_2$ PIV-3 specific HAI and PIV-3/RSV neutralizing titers were equivalent to the titres obtained with live PIV-3 and RSV. Thus, a single inoculation of the chimeric protein was sufficient to elicit neutralizing antibodies against both PIV-3 and RSV. Strong neutralizing PIV-3 and RSV titres also were observed following the booster dose (5 week bleed). These results provide additional evidence that both the RSV and PIV-3 components of the chimeric protein are highly immunogenic.

To assess the ability of the chimeric immunogen to simultaneously protect animals against both RSV and PIV-3, four cotton rats from each group were challenged intranasally with 100 $TCID_{50}$ units of either PIV-3 or RSV. Animals were killed 4 days after virus challenge. Virus titers were determined in lung homogenates. As shown in Table 3 below, animals immunized with either 1 or 10 μg of the chimeric F_{RSV}-HN_{PIV-3} protein were completely protected against challenge with either PIV-3 or RSV. These results provide evidence that the chimeric protein

is not only highly immunogenic but can also simultaneously protect cotton rats against disease caused by both PIV-3 and RSV infection.

Example 15:

This Example illustrates the construction of a Bluescript M13-SK vector containing the chimeric $F_{PIV\text{-}3}$-G_{RSV} gene. This chimeric gene construct contains the 5′ untranslated region of a mutated PIV-3 F gene but lacks the nucleotide sequence coding for the hydrophobic anchor and cytoplasmic tail domains of both a mutated PIV-3 F and the native RSV G genes. The steps involved in constructing this plasmid are outlined in Figures 17 and 18.

The first step (Fig. 17) involved in preparing the PIV-3 F component of the chimeric $F_{PIV\text{-}3}$-G_{RSV} gene construct was to eliminate the putative pre-termination sites within the 18 nucleotide long sequence 5′ CAAGAAAAAGGAATAAAA 3′ (SEQ ID No: 17) located between positions 857 and 874 of the non PCR-amplified PIV-3 F gene and positions 847 and 864 of the PCR-amplified PIV-3 F gene (see Figure 1). To this end, the PIV-F cDNA of the non-PCR amplified PIV-3 F gene was cut at the BsaAI and EcoRI sites. The BsaAI-EcoRI PIV F gene fragment was cloned into the EcoRI site of a Bluescript M13-SK vector using an EcoRI-BsaAI linker. The 857-874 target region of the PIV-3 F gene (non-PCR amplified) then was mutated by oligonucleotide-mediated mutagenesis using the method of Morinaga et al. [1984, Biotechnology 2: 636-639]. Plasmid pPI3Fc (Example 1) was cut with ScaI in the Amp^r gene and dephosphorylated with alkaline phosphatase (plasmid #1). A second sample of plasmid pPI3Fc was cut with BstEII and NsiI to produce a 3.9 Kb restricted plasmid, lacking the 0.9 Kb BstEII-NsiI fragment of the PIV-3 F gene (plasmid #2). A mutagenic 78-mer synthetic oligonucleotide (#2721 shown in Fig. 17-SEQ ID No: 18)) containing the sequence 5′ CAGGAGAAGGGTATCAAG 3′ (SEQ ID

No: 19) was synthesized to specifically mutate the 857-874 DNA segment without changing the F protein sequence. This oligonucleotide was added to plasmid DNAs #1 and #2, denatured at 100°C for 3 min. and renatured by gradual cooling. The mixture then was incubated in the presence of DNA polymerase, dNTPs and T4 ligase and transformed into HB101 cells. Bacteria containing the 1.8 Kb mutated PIV-3 F gene were isolated on YT agar plates containing 100 μg/ml ampicillin. Hybridization with the oligonucleotide probe 5′ AGGAGAAGGGTATCAAG 3′ (SEQ ID No: 20) was used to confirm the presence of the mutated PIV-3 F gene. The mutated gene sequence was confirmed by DNA sequencing. The plasmid containing the mutated PIV-3 gene was designated pPI3Fm.

The second step (Fig. 18) in the engineering of the chimeric gene construct involved constructing a Bluescript based vector to contain the truncated PIV-3 Fm gene lacking the nucleotide sequence coding for the transmembrane anchor domain and cytoplasmic tail of the PIV-3 F protein linked in tandem with the RSV G gene lacking both the 5′ leader sequence and the nucleotide sequence coding for the transmembrane anchor domain and cytoplasmic tail of the G glycoprotein.

To prepare this chimeric gene, the orientation of the mutated PIV-F gene in plasmid pPI3Fm first was reversed by EcoRI digestion and religation to generate plasmid pPI3Fmr. To prepare the PIV-3 F gene component of the chimeric gene, plasmid pPI3Fmr was cut with NotI and BsrI to release the 1.7 Kb truncated PIV-3 F gene. To prepare the RSV G component, the 0.95 Kb RSV-G gene lacking both the 5′ leader sequence and the DNA segment encoding the G protein anchor domain and cytoplasmic tail was released from plasmid pRSVG (Example 1) by cutting the polylinker with EcoRI and the gene with BamHI. The 0.95 Kb EcoRI-BamHI RSV G gene fragment was subcloned into the EcoRI-BamHI sites of a restricted Bluescript

vector, pM13-SK, to produce plasmid pRSVGt. The 0.95 Kb EcoRI-BamHI G gene fragment and the 1.5 Kb NotI-BsrI truncated PIV-3 F gene were linked via a BsrI-BamHI oligonucleotide cassette (SEQ ID No: 9) restoring the F and G gene coding sequences and cloned into the pRSVGt vector restricted with BamHI and NotI in a 3-way ligation. The plasmid thus generated was designated pFG.

Example 16:

This Example outlines the construction of the pD2 baculovirus transfer vector (described in Example 8) containing the chimeric F_{PIV-3}-G_{RSV} gene consisting of a mutated PIV-3 F gene lacking the hydrophobic anchor and cytoplasmic coding regions linked to the RSV G gene lacking both the 5′ leader sequence and the nucleotide sequences encoding the transmembrane anchor domain and cytoplasmic tail of the G protein.

To prepare this construct, plasmid pFG (Example 15) was cut with EcoRI to release the 2.6 Kb F_{PIV-3}-G_{RSV} chimeric gene. The 2.6 Kb EcoRI restricted chimeric gene fragment then was sub-cloned into the EcoRI site of the dephosphorylated pD2 vector to generate the 12.1 Kb plasmid pD2F-G (ATCC 75389).

Example 17:

This Example outlines the preparation of plaque-purified recombinant baculoviruses containing the chimeric F_{PIV-3}-G_{RSV} gene.

Spodoptera frugiperda (Sf9) cells were co-transfected with 2 ug of pD2F-G plasmid DNA (Example 16) and 1 ug of linear wild-type AcNPV DNA (obtained from Invitrogen). Recombinant baculoviruses containing the F_{PIV-3}-G_{RSV} gene were plaque-purified twice according to the procedure outlined in Example 10.

Example 18:

This Example illustrates the presence of the chimeric F_{PIV-3}-G_{RSV} protein in the supernatant of Sf9 and High 5 cells infected with the recombinant baculoviruses.

Sf9 and High 5 cells were infected with recombinant baculoviruses containing the F_{PIV-3}-G_{RSV} gene (Example 16) at a m.o.i. of 5 to 10 pfu/cell. The supernatant of cells infected with the recombinant viruses tested positive for expressed protein in the PIV-3 F specific ELISA. Supernatants of infected cells reacted with both anti-F PIV-3 and anti-G RSV monoclonal antibodies in immunoblots. These results confirm the presence of the chimeric F_{PIV-3}-G_{RSV} protein in the supernatants of infected Sf9 and High 5 cells.

Example 19:

This Example outlines the preparation of recombinant vaccinia viruses expressing the F_{PIV-3} - F_{RSV} and F_{RSV} - HN_{PIV-3} genes.

Vaccinia virus recombinant viruses expressing the F_{PIV-3}-F_{RSV} (designated vP1192) and F_{RSV}-HN_{PIV-3} (designated vP1195) genes were produced at Virogenetics Corporation (Troy, NY) (an entity related to assignee hereof) using the COPAK host-range selection system. Insertion plasmids used in the COPAK host-range selection system contained the vaccinia K1L host-range gene [Perkus et al., (1990) Virology 179:276-286] and the modified vaccinia H6 promoter [Perkus et al. (1989), J. Virology 63:3829-3836]. In these insertion plasmids, the K1L gene, H6 promoter and polylinker region are situated between Copenhagen strain vaccinia flanking arms replacing the ATI region [open reading frames (ORFs) A25L, A26L; Goebel et al., (1990), Virology 179: 247-266; 517-563]. COPAK insertion plasmids are designed for use in *in vivo* recombination using the rescue virus NYVAC (vP866) (Tartaglia et al., (1992) Virology 188: 217-232). Selection of recombinant viruses was done on rabbit kidney cells.

Recombinant viruses, vP1192 and vP1195 were generated using insertion plasmids pES229A-6 and PSD.RN, respectively. To prepare plasmid pES229A-6 containing

the F_{PIV-3}-F_{RSV} gene, the COPAK-H6 insertion plasmid pSD555 was cut with SmaI and dephosphorylated with intestinal alkaline phosphatase. The 2.6 Kb F_{PIV-3}-F_{RSV} gene was retrieved from plasmid pES60A (Example 4) by cutting the plasmid with EcoRI and BamHI. The 2.6 Kb EcoRI-BamHI F_{PIV-3}-F_{RSV} gene was blunt ended with Klenow polymerase, isolated from a low melting point agarose gel and cloned into the SmaI site of the COPAK-H6 insertion plasmid pSD555 to generate plasmid pES229A-6. This positioned the F_{PIV-3}-F_{RSV} ORF such that the 5' end is nearest the H6 promoter.

To prepare plasmid PSD.RN, the pSD555 vector first was cut with SmaI and BamHI. Plasmid pM RF-HN (Example 9) containing the truncated F_{RSV}-HN_{PIV-3} gene was cut with ClaI, blunt ended with Klenow polymerase and then cut with BamHI. The 3.3 Kb F_{RSV}-HN_{PIV-3} gene was cloned into the SmaI-BamHI sites of the pSD555 vector to generate plasmid PSD.RN. This positioned the F_{RSV}-HN_{PIV-3} ORF such that the H6 5' end is nearest the H6 promoter.

Plasmids pES229A-6 and PSD.RN were used in <u>in vitro</u> recombination experiments in vero cells with NYVAC (vP866) as the rescuing virus. Recombinant progeny virus was selected on rabbit kidney (RK)-13 cells (ATCC #CCL37). Several plaques were passaged two times on RK-13 cells. Virus containing the chimeric genes were confirmed by standard in situ plaque hybridization [Piccini et al. (1987), Methods in Enzymology, <u>153</u>:545-563] using radiolabeled probes specific for the PIV and RSV inserted DNA sequences. Plaque purified virus containing the F_{PIV-3}-F_{RSV} and F_{RSV}-HN_{PIV-3} chimeric genes were designated vP1192 and vP1195, respectively.

Radioimmunoprecipitation was done to confirm the expression of the chimeric genes in vP1192 and vP1195 infected cells. These assays were performed with lysates prepared from infected Vero cells [according to the procedure of Taylor et al., (1990) J. Virology <u>64</u>, 1441-

1450] using guinea pig monospecific PIV-3 anti-HN and anti-F antiserum and rabbit anti-RSV F antiserum. Both the anti-PIV F and anti-RSV F antisera precipitated a protein with an apparent molecular weight of approximately 90 koa from vP1192 infected Vero cells. Both anti-RSV F and guinea pig anti-PIV HN antisera precipitated a protein with an apparent molecular weight of approximately 100 kDa from vP1195 infected cells. These results confirmed the production of the $F_{PIV\text{-}3}$-F_{RSV} and F_{RSV}-$HN_{PIV\text{-}3}$ chimeric proteins in Vero cells infected with the recombinant poxviruses.

SUMMARY OF DISCLOSURE

In summary of the disclosure, the present invention provides multimeric hybrid genes which produce chimeric proteins capable of eliciting protection against infection by a plurality of pathogens, particularly PIV and RSV. Modifications are possible within the scope of this invention.

Table 1 Secondary antibody response of guinea pigs immunized with the chimeric F_{RSV}-HN_{PIV-3} protein

Antigen Formulation	Dose (ug)	HAI Titre[a] ($\log_2 \pm$ s.e.)		Neutralization Titre[b] ($\log_2 \pm$ s.e.)			
		PIV-3		PIV-3		RSV	
		6 wk Bleed	8 wk Bleed	6 wk Bleed	8 wk Bleed	6 wk Bleed	8 wk Bleed
Buffer	-	<1.0 ± 0.0	<1.0 ± 0.0	<1.0 ± 0.0	<1.0 ± 0.0	<1.0 ± 0.0	<1.0 ± 0.0
F_{RSV}-HN_{PIV-3}	10.0	9.1 ± 0.3	9.1 ± 0.3	7.1 ± 0.3	7.1 ± 0.5	5.5 ± 0.9	4.5 ± 1.2
	1.0	7.0 ± 2.0	7.3 ± 2.2	5.0 ± 1.5	4.5 ± 1.4	4.5 ± 0.5	3.0 ± 1.0
Live PIV-3		8.6 ± 0.7	7.3 ± 0.6	7.0 ± 0.4	7.3 ± 0.6	N/A	N/A
Live RSV		N/A[c]	N/A	N/A	N/A	5.5 ± 1.5	5.0 ± 1.0

[a] Reciprocal mean $\log_2$ serum dilution which inhibits erythrocyte agglutination by 4 hemagglutinating units of PIV-3

[b] Reciprocal mean $\log_2$ serum dilution which blocks hemadsorption of 100 $TCID_{50}$ units of PIV-3 or RSV

[c] N/A - not applicable

Table 2: Serum antibody response of cotton rats immunized with the chimeric F_{RSV}-$HN_{PIV\text{-}3}$ protein[a]

Antigen Formulation	Dose (ug)	HAI Titre[b] ($\log_2$ ± s.d.)		Neutralization Titre[c] ($\log_2$ ± s.d.)			
		PIV-3		PIV-3		RSV	
		4 wk Bleed	5 wk Bleed	4 wk Bleed	5 wk Bleed	4 wk Bleed	5 wk Bleed
Buffer	-	2.8 ± 0.5	<3.0 ± 0.0	<1.0 ± 1.0	<1.0 ± 0.0	1.8 ± 0.3	0.8 ± 0.7
F_{RSV}-$HN_{PIV\text{-}3}$	10.0	9.5 ± 1.3	10.5 ± 0.6	>9.0 ± 0.0	>9.0 ± 0.0	5.2 ± 1.1	5.8 ± 0.9
	1.0	9.3 ± 1.0	10.3 ± 0.5	>9.0 ± 0.0	>9.0 ± 0.0	5.0 ± 0.7	5.8 ± 1.2
Live PIV-3		7.0 ± 0.0	8.5 ± 0.7	>9.0 ± 0.0	9.2 ± 0.7	N/A	N/A
Live RSV		N/A[d]	N/A	N/A	N/A	5.5 ± 0.6	8.5 ± 0.6

[a] Each value represents the mean titre of antisera from 8 animals.

[b] Reciprocal mean $\log_2$ serum dilution which inhibits erythrocyte agglutination by 4 hemagglutinating units of PIV-3

[c] Reciprocal mean $\log_2$ serum dilution which blocks hemadsorption of 100 $TCID_{50}$ units of PIV-3 or RSV

[d] N/A - not applicable

Table 3. Response of immunized cotton rats to PIV/RSV challenge[a]

Antigen Formulation	Dose (ug)	Mean virus lung titre $\log_{10}$/g lung ± s.d. RSV	PIV-3
Buffer	-	3.7 ± 0.3	3.4 ± 0.3
F_{RSV}-HN_{PIV-3}	10.0	≤1.5 ± 0.0	≤1.5 ± 0.0
F_{RSV}-HN_{PIV-3}	1.0	≤1.5 ± 0.0	≤1.5 ± 0.0
Live RSV		≤1.5 ± 0.0	≤1.5 ± 0.0
Live PIV-3		≤1.5 ± 0.0	≤1.5 ± 0.0

[a] Animals were challenged intranasally with 100 $TCID_{50}$ units of PIV-3 or RSV and killed 4 days later. Each value represents the mean virus lung titre of 4 animals.

CLAIMS

What we claim is:

1. A multimeric hybrid gene, comprising a gene sequence coding for an antigenic region of a protein from a first pathogen linked to a gene sequence coding for an antigenic region of a protein from a second pathogen.
2. The hybrid gene of claim 1 wherein said first and second pathogens are selected from bacterial and viral pathogens.
3. The hybrid gene of claim 2 wherein both said first and second pathogens are viral pathogens.
4. The hybrid gene of claim 1 wherein said first and second pathogens are selected from those causing different respiratory tract diseases.
5. The hybrid gene of claim 4 wherein said first and second pathogens causing different respiratory tract diseases are selected from the paramoxyviridae family of viruses.
6. The hybrid gene of claim 1 wherein at least one of said gene sequences is mutated while retaining antigenicity.
7. The hybrid gene of claim 6 wherein said mutation is at a putative pre-termination site.
8. The hybrid gene of claim 1 wherein said first pathogen is parainfluenza virus (PIV) and said second pathogen is respiratory syncytial virus (RSV).
9. The hybrid gene of claim 1, comprising at least one gene sequence coding for a parainfluenza virus (PIV) protein linked to at least one gene sequence coding for a respiratory syncytial virus (RSV) protein.
10. The hybrid gene of claim 9, wherein said parainfluenza virus protein is selected from PIV-3 F and HN proteins and said respiratory syncytial virus protein is selected from RSV G and F proteins.
11. The hybrid gene of claim 1 consisting of a gene sequence coding for a human PIV-3 F or HN protein or an

immunogenic epitope-containing fragment thereof linked to a gene sequence coding for a human RSV G or F protein or an immunogenic epitope-containing fragment thereof.

12. The hybrid gene of claim 11 which is selected from F_{PIV-3} - F_{RSV}, F_{RSV} - HN_{PIV-3} and F_{PIV-3} - G_{RSV} hybrid genes.

13. The hybrid gene of clain 1 contained in an expression vector.

14. The hybrid gene of claim 13 in the form of plasmid pAC DR7, pD2 RF-HN or pD2 F-G.

15. The hybrid gene of claim 1 further comprising at least one gene encoding at least one immunogenic and/or immunostimulating molecule.

16. Cells containing the multimeric hybrid gene of claim 1 for expression of a chimeric protein encoded by said gene.

17. The cells of claim 16 which are bacterial cells, mammalian cells, insect cells, yeast cells or fungal cells.

18. A chimeric protein, comprising an antigenic region of a protein from a first pathogen linked to an antigenic region of a protein from a second pathogen.

19. The protein of claim 18, wherein said first and second pathogens are selected from bacterial and viral pathogens.

20. The protein of claim 19 wherein both said first and second pathogens are viral pathogens.

21. The protein of claim 18, wherein said first and second pathogens are selected from those causing different respiratory tract diseases.

22. The protein of claim 21 wherein said first and second pathogens causing different respiratory tract diseases are selected from the paramoxyviridae family of viruses.

23. The protein of claim 18, wherein said first pathogen is parainfluenza virus (PIV) and said second pathogen is respiratory syncytial virus (RSV).

24. The protein of claim 18 comprising at least one parainfluenza virus (PIV) protein linked to at least one respiratory syncytial virus (RSV) protein.
25. The protein of claim 24, wherein said PIV protein is selected from PIV-3 F and HN proteins and said RSV protein is selected from RSV G and F proteins.
26. The protein of claim 18 consisting of a human parainfluenza virus-3 (PIV-3) F or HN protein or an immunogenic epitope-containing fragment thereof linked to a human respiratory syncytial virus (RSV) G or F protein or an immunogenic epitope-containing fragment thereof.
27. The protein of claim 26 which is selected from $F_{PIV-3} - F_{RSV}$, $F_{RSV} - HN_{PIV-3}$ and $F_{PIV-3} - G_{RSV}$ chimeric proteins.
28. A process for preparation of a chimeric protein which comprises:

isolating a gene sequence coding for an antigenic region of a protein from a first pathogen,

isolating a gene sequence coding for an antigenic region of a protein from a second pathogen,

linking said gene sequences to form a multimeric

hybrid gene, and expressing the multimeric hybrid gene in a cellular expression system

29. The process of claim 28 wherein said multimeric hybrid gene comprises a gene sequence coding for a PIV-F or HN protein or an immunogenic epitope-containing fragment thereof linked to a gene sequence coding for a human RSV G or F protein or an epitope-containing fragment thereof.
30. The process of claim 29 wherein said multimeric hybrid gene is selected from $F_{PIV-3} - F_{RSV}$, $F_{RSV} - HN_{PIV-3}$ and $F_{PIV-3} - G_{RSV}$ hybrid genes.
31. The process of claim 30 wherein said multimeric hybrid gene is contained in an expression vector comprising plasmid pAC QR7, pD2 RF-HN or pD2 F-G.
32. The process of claim 28 wherein said cellular expression system is provided by bacterial cells,

mammalian cells, insect cells, yeast cells or fungal cells.

33. The process of claim 32 including separating a chimeric protein from a culture of said cellular expression system and purifying the separated chimeric protein.

34. A live vector for antigen delivery containing the gene of claim 1.

35. The live vector of claim 34 which is a viral vector.

36. The live vector of claim 35 wherein said viral vector is selected from poxviral, adenoviral and retroviral viral vectors.

37. The live vector of claim 34 which is a bacterial vector.

38. The live vector of claim 37 wherein said bacterial vector is selected from salmonella and mycobacteria.

39. A vaccine against diseases caused by multiple pathogenic infections, comprising a chimeric protein comprising an antigen region of a protein from a first pathogen linked to an antigenic region of a protein from a second pathogen, and a physiologically-acceptable carrier therefor.

40. The vaccine of claim 39, wherein said first and second pathogens are selected from bacterial and viral pathogens.

41. The vaccine of claim 39, which also contains at least one other immunogenic and/or immunostimulating molecule.

42. The vaccine of claim 40 wherein both said first and second pathogens are viral pathogens.

43. The vaccine of claim 39, wherein said first and second pathogens are selected from those causing upper and lower respiratory tract diseases.

44. The vaccine of claim 39, wherein said first pathogen is parainfluenza virus (PIV) and said second pathogen is respiratory syncytial virus (RSV).

45. The vaccine of claim 39 against infection by both parainfluenza virus (PIV) and respiratory syncytial virus (RSV), comprising a recombinant multimeric protein containing at least one segment consisting of a PIV protein or an immunogenic epitope-containing fragment thereof linked to at least one segment consisting of a RSV protein or an immunogenic epitope-containing fragment thereof, and a carrier therefor.

46. The vaccine of claim 45 wherein said recombinant multimeric protein is a recombinant chimeric protein containing a segment consisting of a PIV-3 F or HN protein or an immunogenic epitope-containing fragment thereof linked to a segment consisting of an RSV G or F protein or an immunogenic epitope-containing fragment thereof.

47. The vaccine of claim 46 containing at least one additional protein of PIV or RSV or chimeric protein thereof.

48. The vaccine of claim 39 wherein said carrier comprises an adjuvant.

49. The vaccine of claim 39 wherein said carrier is an ISCOM, a liposome or a microparticle.

50. The vaccine of claim 46 formulated to be administered in an injectable form, intranasally or orally.

51. The vaccine of claim 39 further comprising means for delivering said multimeric protein specifically to cells of the immune system.

52. The vaccine of claim 51 wherein said delivery means comprises a toxin molecule or an antibody.

53. A vaccine against diseases caused by multiple pathogenic infection, comprising a live vector as claimed in claim 34, and a physiologically-acceptable carrier therefor.

54. A method of immunizing a host against diseases caused by multiple pathogenic infections, which comprises

administering to a host an effective amount of a vaccine as claimed in claim 28 or 53.

55. The method of claim 54 wherein said vaccine is against diseases caused by parainfluenza virus (PIV) and respiratory syncytial virus (RSV).

56. The method of claim 55 wherein said host is selected from infants, young children, pregnant women, women of child-bearing age and susceptible persons.

57. A diagnostic reagent for detecting infection by a plurality of different pathogens in a host, comprising the chimeric protein claimed in claim 18.

58. A method of detecting infection by a plurality of different pathogens in a host, which comprises using said chimeric protein claimed in claim 18.

FIG.1A. NUCLEOTIDE SEQUENCE OF THE PIV-3 F GENE (PCR-AMPLIFIED)

```
AAGTCAATACCAACAACTATTAGCAGTCATACGTGCAAGAACAAGAAAGAAGAGATTCAA
TTCAGTTATGGTTGTTGATAATCGTCAGTATGCACGTTCTTGTTCTTTCTTCTCTAAGTT
         10        20        30        40        50        60

AAAGCTAAATAAGAGAAATCAAAACAAAAGGTATAGAACACCCGAACAACAAAATCAAAA
TTTCGATTTATTCTCTTTAGTTTTGTTTTCCATATCTTGTGGGCTTGTTGTTTTAGTTTT
         70        80        90       100       110       120

CATCCAATCCATTTTAAACAAAAATTCCAAAAGAGACCGGCAACACAACAAGCACCAAAC
GTAGGTTAGGTAAAATTTGTTTTTAAGGTTTTCTCTGGCCGTTGTGTTGTTCGTGGTTTG
        130       140       150       160       170       180
  <-------------------- SP --------------------->
   MET PRO THR [LEU] ILE LEU LEU ILE ILE THR THR MET ILE MET ALA [SER] SER CYS GLN
ACAATGCCAACTTTAATACTGCTAATTATTACAACAATGATTATGGCATCTTCCTGCCAA
TGTTACGGTTGAAATTATGACGATTAATAATGTTGTTACTAATACCGTAGAAGGACGGTT
        190       200       210       220       230       240

ILE ASP ILE THR LYS LEU GLN HIS VAL GLY VAL LEU VAL ASN SER PRO LYS GLY MET LYS
ATACATATCACAAAACTACAGCATGTAGGTGTATTGGTCAACAGTCCCAAAGGGATGAAG
TATGTATAGTGTTTTGATGTCGTACATCCACATAACCAGTTGTCAGGGTTTCCCTACTTC
        250       260       270       280       290       300

ILE SER GLN ASN PHE GLU THR ARG TYR LEU ILE LEU SER LEU ILE PRO LYS ILE GLU ASP
ATATCACAAAACTTCGAAACAAGATATCTAATTTTGAGCCTCATACCAAAAATAGAAGAC
TATAGTGTTTTGAAGCTTTGTTCTATAGATTAAAACTCGGAGTATGGTTTTTATCTTCTG
        310       320       330       340       350       360

SER ASN SER CYS GLY ASP GLN GLN ILE LYS GLN TYR LYS ARG LEU LEU ASP ARG LEU ILE
TCTAACTCTTGTGGTGACCAACAGATCAAACAATACAAGAGGTTATTGGATAGACTGATC
AGATTGAGAACACCACTGGTTGTCTAGTTTGTTATGTTCTCCAATAACCTATCTGACTAG
        370       380       390       400       410       420
```

FIG.1B.

```
ILE PRO LEU TYR ASP GLY LEU ARG LEU GLN LYS ASP VAL ILE VAL [THR] ASN GLN GLU SER
ATCCCTCTATATGATGGATTAAGATTACAGAAAGATGTGATAGTAACCAATCAAGAATCC
TAGGGAGATATACTACCTAATTCTAATGTCTTTCTACACTATCATTGGTTAGTTCTTAGG
430 440 450 460 470 480

F2-F1 CLEAVAGE SITE
ASN GLU ASN THR ASP PRO ARG THR [ARG] ARG↓[SER] PHE GLY GLY VAL ILE GLY THR ILE ALA
AATGAAAACACTGATCCCAGAACAAGACGATCCTTTGGAGGGGTAATTGGAACCATTGCT
TTACTTTTGTGACTAGGGTCTTGTTCTGCTAGGAAACCTCCCCATTAACCTTGGTAACGA
490 500 510 520 530 540

LEU GLY VAL ALA THR SER ALA GLN ILE THR ALA ALA VAL ALA LEU VAL GLU ALA LYS GLN
CTGGGAGTAGCAACCTCAGCACAAATTACAGCGGCAGTTGCTCTGGTTGAAGCCAAGCAG
GACCCTCATCGTTGGAGTCGTGTTTAATGTCGCCGTCAACGAGACCAACTTCGGTTCGTC
550 560 570 580 590 600

ALA [LYS] SER ASP ILE GLU LYS LEU LYS GLU ALA ILE ARG ASP THR ASN LYS ALA VAL GLN
GCAAAATCACACATCGAAAAACTCAAAGAAGCAATCAGGGACACAAACAAAGCAGTGCAG
CGTTTTAGTGTGTAGCTTTTTGAGTTTCTTCGTTAGTCCCTGTGTTTGTTTCGTCACGTC
610 620 630 640 650 660

SER VAL GLN SER SER ILE GLY ASN LEU ILE VAL ALA ILE LYS SER VAL GLN ASP TYR VAL
TCAGTTCAGAGCTCTATAGGAAATTTAATAGTAGCAATTAAATCAGTCCAAGATTATGTC
AGTCAAGTCTCGAGATATCCTTTAAATTATCATCGTTAATTTAGTCAGGTTCTAATACAG
670 680 690 700 710 720

ASN [ASN] GLU ILE VAL PRO SER ILE ALA ARG LEU GLY CYS GLU ALA ALA GLY LEU GLN LEU
AACAACGAAATCGTGCCATCGATTGCTAGACTAGGTTGTGAAGCAGCAGGACTTCAATTA
TTGTTGCTTTAGCACGGTAGCTAACGATCTGATCCAACACTTCGTCGTCCTGAAGTTAAT
730 740 750 760 770 780

GLY ILE ALA LEU THR GLN HIS TYR SER GLU LEU THR ASN ILE PHE GLY ASP ASN ILE GLY
GGAATTGCATTAACACAGCATTACTCAGAATTAACAAACATATTTGGTGATAACATAGGA
CCTTAACGTAATTGTGTCGTAATGAGTCTTAATTGTTTGTATAAACCACTATTGTATCCT
790 800 810 820 830 840
```

FIG.1C.

```
SER LEU GLN GLU LYS GLY ILE LYS LEU GLN GLY ILE ALA SER LEU TYR ARG THR ASN ILE
TCGTTACAAGAAAAAGGAATAAAATTACAAGGTATAGCATCATTATACCGCACAAATATC
AGCAATGTTCTTTTTCCTTATTTTAATGTTCCATATCGTAGTAATATGGCGTGTTTATAG
        850       860       870       880       890       900

THR GLU ILE PHE THR THR SER THR VAL ASP LYS TYR ASP ILE TYR ASP LEU LEU PHE THR
ACAGAAATATTCACAACATCAACAGTTGATAAATATGATATCTATGATCTATTATTTACA
TGTCTTTATAAGTGTTGTAGTTGTCAACTATTTATACTATAGATACTAGATAATAAATGT
        910       920       930       940       950       960

GLU SER ILE LYS VAL ARG VAL ILE ASP VAL ASP LEU ASN ASP TYR SER ILE THR LEU GLN
GAATCAATAAAGGTGAGAGTTATAGATGTTGATTTGAATGATTACTCAATCACCCTCCAA
CTTAGTTATTTCCACTCTCAATATCTACAACTAAACTTACTAATGAGTTAGTGGGAGGTT
        970       980       990      1000      1010      1020

VAL ARG LEU PRO LEU LEU THR ARG LEU LEU ASN THR GLN ILE TYR LYS VAL ASP SER ILE
GTCAGACTCCCTTTATTAACTAGGCTGCTGAACACTCAGATCTACAAAGTAGATTCCATA
CAGTCTGAGGGAAATAATTGATCCGACGACTTGTGAGTCTAGATGTTTCATCTAAGGTAT
       1030      1040      1050      1060      1070      1080

SER TYR ASN ILE GLN ASN ARG GLU TRP TYR ILE PRO LEU PRO SER HIS ILE MET THR LYS
TCATATAATATCCAAAACAGAGAATGGTATATCCCTCTTCCCAGCCATATCATGACGAAA
AGTATATTATAGGTTTTGTCTCTTACCATATAGGGAGAAGGGTCGGTATAGTACTGCTTT
       1090      1100      1110      1120      1130      1140

GLY ALA PKE LEU GLY GLY ALA ASP VAL LYS GLU CYS ILE GLU ALA PHE SER SER TYR ILE
GGGGCATTTCTAGGTGGAGCAGATGTCAAGGAATGTATAGAAGCATTCAGCAGTTATATA
CCCCGTAAAGATCCACCTCGTCTACAGTTCCTTACATATCTTCGTAAGTCGTCAATATAT
       1150      1160      1170      1180      1190      1200

CYS PRO SER ASP PRO GLY PHE VAL LEU ASN HIS GLU KET GLU SER CYS LEU SER GLY ASN
TGCCCTTCTGATCCAGGATTTGTACTAAACCATGAAATGGAGAGCTGCTTATCAGGAAAC
ACGGGAAGACTAGGTCCTAAACATGATTTGGTACTTTACCTCTCGACGAATAGTCCTTTG
       1210      1220      1230      1240      1250      1260
```

FIG.1D.

ILE SER GLN CYS PRO ARG THR $\boxed{\text{THR}}$ VAL $\boxed{\text{THR}}$ SER ASP ILE VAL PRO ARG TYR ALA PHE VAL
ATATCCCAATGTCCAAGAACCACGGTCACATCAGACATTGTTCCAAGATATGCATTCGTC
TATAGGGTTACAGGTTCTTGGTGCCAGTGTAGTCTGTAACAAGGTTCTATACGTAAGCAG
1270 1280 1290 1300 1310 1320

ASN GLY GLY VAL VAL ALA ASN CYS ILE THR THR THR CYS THR CYS ASN GLY ILE $\boxed{\text{ASP}}$ ASN
AATGGAGGAGTGGTTGCAAACTGTATAACAACCACCTGTACATGCAACGGAATCGACAAT
TTACCTCCTCACCAACGTTTGACATATTGTTGGTGGACATGTACGTTGCCTTAGCTGTTA
1330 1340 1350 1360 1370 1380

ARG ILE ASN GLN PRO PRO ASP GLN GLY VAL LYS ILE ILE THR HIS LYS GLU CYS ASN THR
AGAATCAATCAACCACCTGATCAAGGAGTAAAAATTATAACACATAAAGAATGTAATACA
TCTTAGTTAGTTGGTGGACTAGTTCCTCATTTTTAATATTGTGTATTTCTTACATTATGT
1390 1400 1410 1420 1430 1440

ILE GLY ILE ASN GLY MET LEU PHE ASN THR ASN LYS GLU GLY THR LEU ALA PHE TYR THR
ATAGGTATCAACGGAATGCTGTTCAATACAAATAAAGAAGGAACTCTTGCATTCTACACA
TATCCATAGTTGCCTTACGACAAGTTATGTTTATTTCTTCCTTGAGAACGTAAGATGTGT
1450 1460 1470 1480 1490 1500

PRO ASN ASP ILE THR LEU ASN ASN SER VAL ALA LEU ASP PRO ILE ASP ILE SER ILE GLU
CCAAATGATATAACACTAAATAATTCTGTTGCACTTGATCCAATTGACATATCAATCGAG
GGTTTACTATATTGTGATTTATTAAGACAACGTGAACTAGGTTAACTGTATAGTTAGCTC
1510 1520 1530 1540 1550 1560

LEU ASN LYS ALA LYS SER ASP LEU GLU GLU SER LYS GLU TRP ILE ARG ARG SER ASN GLN
CTTAACAAAGCCAAATCAGATCTAGAAGAATCAAAAGAATGGATAAGAAGGTCAAATCAA
GAATTGTTTCGGTTTAGTCTAGATCTTCTTAGTTTTCTTACCTATTCTTCCAGTTTAGTT
1570 1580 1590 1600 1610 1620

TM

LYS LEU ASP SER ILE GLY ASN TRP HIS GLN SER SER THR THR ILE ILE ILE $\boxed{\text{ILE}}$ LEU ILE
AAACTAGATTCTATTGGAAACTGGCATCAATCTAGCACTACAATCATAATTATTTTAATA
TTTGATCTAAGATAACCTTTGACCGTAGTTAGATCGTGATGTTAGTATTAATAAAATTAT
1630 1640 1650 1660 1670 1680

SUBSTITUTE SHEET

FIG.1E

```
MET ILE ILE ILE LEU PHE ILE ILE ASN VAL THR ILE ILE [THR] ILE ALA [ILE] LYS TYR TYR
ATGATCATTATATTGTTTATAATTAATGTAACGATAATTACAATTGCAATTAAGTATTAC
TACTAGTAATATAACAAATATTAATTACATTGCTATTAATGTTAACGTTAATTCATAATG
         1690      1700      1710      1720      1730      1740

ARG ILE GLN LYS ARG ASN ARG VAL ASP GLN ASN ASP LYS PRO TYR VAL LEU THR ASN LYS
AGAATTCAAAAGAGAAATCGAGTGGATCAAAATGACAAGCCATATGTACTAACAAACAAA
TCTTAAGTTTTCTCTTTAGCTCACCTAGTTTTACTGTTCGGTATACATGATTGTTTGTTT
         1750      1760      1770      1780      1790      1800

TGACATATCTATAGATCATTAGATATTAAAATTATAAAAAACTT
ACTGTATAGATATCTAGTAATCTATAATTTTAATATTTTTTGAA
         1810      1820      1830      1840
```

NUCLEOTIDE SEQUENCE OF THE PIV-3 F GENE. THE cDNA SEQUENCE IS SHOWN IN THE PLUS (mRNA) STRAND SENSE IN THE 5' TO 3' DIRECTION. THE SIGNAL PEPTIDE (SP) AND THE TRANSMEMBRANE (TM) ANCHOR DOMAIN ARE UNDERLINED. THE PREDICTED F2-F1 CLEAVAGE SITE IS INDICATED BY THE ARROW (↓). AMINO ACIDS DIFFERING FROM THE PUBLISHED PRIMARY SEQUENCE OF THE PROTEIN ENCODED BY THE PIV-3 F GENE ARE BOXED.

RESTRICTION MAP OF THE PIV-3 F GENE

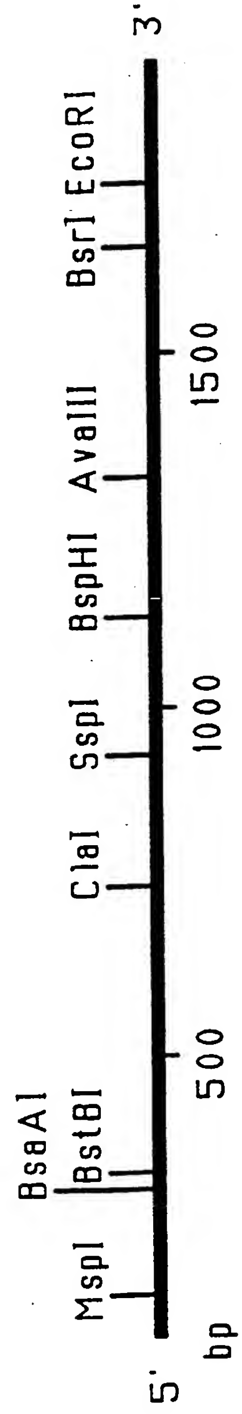

FIG.2.

FIG.3A.

NUCLEOTIDE SEQUENCE OF THE PIV-3 HN GENE.

```
                        MET GLU TYR TRP LYS HIS THR ASN HIS GLY LYS ASP ALA GLY
5' AGACAAATCCAAATTCGAGATGGAATACTGGAAGCATACCAATCACGGAAAGGATGCTGG
   TCTGTTTAGGTTTAAGCTCTACCTTATGACCTTCGTATGGTTAGTGCCTTTCCTACGACC
            10        20        30        40        50        60
                                                          <--TM--

ASN GLU LEU GLU THR SER MET ALA THR [ASN] GLY ASN LYS [LEU] THR ASN LYS ILE THR TYR
   CAATGAGCTGGAGACGTCCATGGCTACTAATGGCAACAAGCTCACCAATAAGATAACATA
   GTTACTCGACCTCTGCAGGTACCGATGATTACCGTTGTTCGAGTGGTTATTCTATTGTAT
            70        80        90       100       110       120
   ------------------------------------------------------------>

ILE LEU TRP THR ILE ILE LEU VAL LEU LEU SER ILE VAL PHE ILE ILE VAL LEU ILE ASN
   TATATTATGGACAATAATCCTGGTGTTATTATCAATAGTCTTCATCATAGTGCTAATTAA
   ATATAATACCTGTTATTAGGACCACAATAATAGTTATCAGAAGTAGTATCACGATTAATT
           130       140       150       160       170       180

SER ILE LYS SER GLU LYS ALA HIS GLU SER LEU LEU GLN ASP [ILE] ASN ASN GLU PHE MET
   TTCCATCAAAAGTGAAAAGGCTCATGAATCATTGCTGCAAGACATAAATAATGAGTTTAT
   AAGGTAGTTTTCACTTTTCCGAGTACTTAGTAACGACGTTCTGTATTTATTACTCAAATA
           190       200       210       220       230       240

GLU [ILE] THR GLU LYS ILE GLN MET ALA SER ASP ASN [THR] ASN ASP LEU ILE GLN SER GLY
   GGAAATTACAGAAAAGATCCAAATGGCATCGGATAATACCAATGATCTAATACAGTCAGG
   CCTTTAATGTCTTTTCTAGGTTTACCGTAGCCTATTATGGTTACTAGATTATGTCAGTCC
           250       260       270       280       290       300

VAL ASN THR ARG LEU LEU THR ILE GLN SER HIS VAL GLN ASN TYR ILE PRO ILE SER LEU
   AGTGAATACAAGGCTTCTTACAATTCAGAGTCATGTCCAGAATTATATACCAATATCACT
   TCACTTATGTTCCGAAGAATGTTAAGTCTCAGTACAGGTCTTAATATATGGTTATAGTGA
           310       320       330       340       350       360
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```
 THR GLN GLN MET SER ASP LEU ARG LYS PHE ILE SER GLU ILE THR ILE ARG ASN ASP ASN
GACACAACAGATGTCAGATCTTAGGAAATTCATTAGTGAAATTACAATTAGAAATGATAA
CTGTGTTGTCTACAGTCTAGAATCCTTTAAGTAATCACTTTAATGTTAATCTTTACTATT
        370       380       390       400       410       420

 GLN GLU VAL LEU PRO GLN ARG ILE THR HIS ASP VAL GLY ILE LYS PRO LEU ASN PRO ASP
TCAAGAAGTGCTGCCACAAAGAATAACACATGATGTGGGTATAAAACCTTTAAATCCAGA
AGTTCTTCACGACGGTGTTTCTTATTGTGTACTACACCCATATTTTGGAAATTTAGGTCT
        430       440       450       460       470       480

 ASP PHE TRP ARG CYS THR SER GLY LEU PRO SER LEU MET LYS THR PRO LYS ILE ARG LEU
TGATTTTTGGAGATGCACGTCTGGTCTTCCATCTTTAATGAAAACTCCAAAAATAAGGTT
ACTAAAAACCTCTACGTGCAGACCAGAAGGTAGAAATTACTTTTGAGGTTTTTATTCCAA
        490       500       510       520       530       540

 MET PRO GLY PRO GLY LEU LEU ALA MET PRO THR THR VAL ASP GLY CYS ILE ARG THR PRO
AATGCCAGGGCCGGGATTATTAGCTATGCCAACGACTGTTGATGGCTGTATCAGAACTCC
TTACGGTCCCGGCCCTAATAATCGATACGGTTGCTGACAACTACCGACATAGTCTTGAGG
        550       560       570       580       590       600

 SER LEU VAL ILE ASN ASP LEU ILE TYR ALA TYR THR SER ASN LEU ILE THR ARG GLY CYS
GTCCTTAGTTATAAATGATCTGATTTATGCTTATACCTCAAATCTAATTACTCGAGGTTG
CAGGAATCAATATTTACTAGACTAAATACGAATATGGAGTTTAGATTAATGAGCTCCAAC
        610       620       630       640       650       660

 GLN ASP ILE GLY LYS SER TYR GLN VAL LEU GLN ILE GLY ILE ILE THR VAL ASN SER ASP
TCAGGATATAGGAAAATCATATCAAGTCTTACAGATAGGGATAATAACTGTAAACTCAGA
AGTCCTATATCCTTTTAGTATAGTTCAGAATGTCTATCCCTATTATTGACATTTGAGTCT
        670       680       690       700       710       720

 LEU VAL PRO ASP LEU ASN PRO ARG ILE SER HIS THR PHE ASN ILE ASN ASP ASN ARG LYS
CTTGGTACCTGACTTAAATCCCAGGATCTCTCATACTTTTAACATAAATGACAATAGGAA
GAACCATGGACTGAATTTAGGGTCCTAGAGAGTATGAAAATTGTATTTACTGTTATCCTT
        730       740       750       760       770       780
```

FIG.3B.

```
SRE CYS SER LEU ALA LEU LEU ASN THR ASP VAL TYR GLN LEU CYS SER THR PRO LYS VAL
GTCATGTTCTCTAGCACTCCTAAATACAGATGTATATCAACTGTGTTCAACTCCCAAAGT
CAGTACAAGAGATCGTGAGGATTTATGTCTACATATAGTTGACACAAGTTGAGGGTTTCA
       790       800       810       820       830       840

ASP GLU ARG SER ASP TYR ALA SER SER GLY ILE GLU ASP ILE VAL LEU ASP ILE VAL ASN
TGATGAAAGATCAGATTATGCATCATCAGGCATAGAAGATATTGTACTTGATATTGTCAA
ACTACTTTCTAGTCTAATACGTAGTAGTCCGTATCTTCTATAACATGAACTATAACAGTT
       850       860       870       880       890       900

TYR ASP GLY SER ILE SER THR THR ARG PHE LYS ASN ASN ASN ILE SER PHE ASP GLN PRO
TTATGATGGCTCAATCTCAACAACAAGATTTAAGAATAATAACATAAGCTTTGATCAACC
AATACTACCGAGTTAGAGTTGTTGTTCTAAATTCTTATTATTGTATTCGAAACTAGTTGG
       910       920       930       940       950       960

TYR ALA ALA LEU TYR PRO SER VAL GLY PRO GLY ILE TYR TYR LYS GLY LYS ILE ILE PHE
TTATGCTGCACTATACCCATCTGTTGGACCAGGGATATACTACAAAGGCAAAATAATATT
AATACGACGTGATATGGGTAGACAACCTGGTCCCTATATGATGTTTCCGTTTTATTATAA
       970       980       990      1000      1010      1020

LEU GLY TYR GLY GLY LEU GLU HIS PRO ILE ASN GLU ASN VAL ILE CYS ASN THR THR GLY
TCTCGGGTATGGAGGTCTTGAACATCCAATAAATGAGAATGTAATCTGCAACACAACTGG
AGAGCCCATACCTCCAGAACTTGTAGGTTATTTACTCTTACATTAGACGTTGTGTTGACC
      1030      1040      1050      1060      1070      1080

CYS PRO GLY LYS THR GLN ARG ASP CYS ASN GLN ALA SER HIS SER PRO TRP PHE SER ASP
GTGTCCCGGGAAAACACAGAGAGACTGCAATCAGGCATCTCATAGTCCATGGTTTTCAGA
CACAGGGCCCTTTTGTGTCTCTCTGACGTTAGTCCGTAGAGTATCAGGTACCAAAAGTCT
      1090      1100      1110      1120      1130      1140

ARG ARG MET VAL ASN SER ILE ILE VAL VAL ASP LYS GLY LEU ASN SER ILE PRO LYS LEU
TAGGAGGATGGTCAACTCTATCATTGTTGTTGACAAAGGCTTAAACTCAATTCCAAAATT
ATCCTCCTACCAGTTGAGATAGTAACAACAACTGTTTCCGAATTTGAGTTAAGGTTTTAA
      1150      1160      1170      1180      1190      1200
```

FIG.3C.

```
 LYS VAL TRP THR ILE SER MET ARG GLN ASN TYR TRP GLY SER GLU GLY ARG LEU LEU LEU
GAAGGTATGGACGATATCTATGAGACAGAATTACTGGGGGTCAGAAGGAAGGTTACTTCT
CTTCCATACCTGCTATAGATACTCTGTCTTAATGACCCCCAGTCTTCCTTCCAATGAAGA
        1210      1220      1230      1240      1250      1260

 LEU GLY ASN LYS ILE TYR ILE TYR THR ARG SER THR SER TRP HIS SER LYS LEU GLN LEU
ACTAGGTAACAAGATCTATATATATACAAGATCCACAAGTTGGCATAGCAAGTTACAATT
TGATCCATTGTTCTAGATATATATATGTTCTAGGTGTTCAACCGTATCGTTCAATGTTAA
        1270      1280      1290      1300      1310      1320

 GLY ILE ILE ASP ILE THR ASP TYR SER ASP ILE ARG ILE LYS TRP THR TRP HIS ASN VAL
AGGAATAATTGATATTACTGATTACAGTGATATAAGGATAAAATGGACATGGCATAATGT
TCCTTATTAACTATAATGACTAATGTCACTATATTCCTATTTTACCTGTACCGTATTACA
        1330      1340      1350      1360      1370      1380

 LEU SER ARG PRO GLY ASN ASN GLU CYS PRO TRP GLY HIS SER CYS PRO ASP GLY CYS ILE
GCTATCAAGACCAGGAAACAATGAATGTCCATGGGGACATTCATGTCCAGATGGATGTAT
CGATAGTTCTGGTCCTTTGTTACTTACAGGTACCCCTGTAAGTACAGGTCTACCTACATA
        1390      1400      1410      1420      1430      1440

 THR GLY VAL TYR THR ASP ALA TYR PRO LEU ASN PRO THR GLY SER ILE VAL SER SER VAL
AACAGGAGTATATACTGATGCATATCCACTCAATCCCACAGGGAGCATTGTGTCATCTGT
TTGTCCTCATATATGACTACGTATAGGTGAGTTAGGGTGTCCCTCGTAACACAGTAGACA
        1450      1460      1470      1480      1490      1500

 ILE LEU ASP SER GLN LYS SER ARG VAL ASN PRO VAL ILE THR TYR SER THR ALA THR GLU
CATATTAGATTCACAAAAATCGAGAGTGAACCCAGTCATAACTTACTCAACAGCAACCGA
GTATAATCTAAGTGTTTTTAGCTCTCACTTGGGTCAGTATTGAATGAGTTGTCGTTGGCT
        1510      1520      1530      1540      1550      1560

 ARG VAL ASN GLU LEU ALA ILE ARG ASN ARG THR LEU SER ALA GLY TYR THR THR THR SER
AAGAGTAAACGAGCTGGCCATCCGAAACAGAACACTCTCAGCTGGATATACAACAACAAG
TTCTCATTTGCTCGACCGGTAGGCTTTGTCTTGTGAGAGTCGACCTATATGTTGTTGTTC
        1570      1580      1590      1600      1610      1620
```

FIG.3D.

```
 CYS ILE THR HIS TYR ASN LYS GLY TYR CYS PHE HIS ILE VAL GLU ILE ASN [GLN] LYS SER
CTGCATCACACACTATAACAAAGGATATTGTTTTCATATAGTAGAAATAAATCAGAAAAG
GACGTAGTGTGTGATATTGTTTCCTATAACAAAAGTATATCATCTTTATTTAGTCTTTTC
          1630      1640      1650      1660      1670      1680

 LEU [ASN] THR [LEU] GLN PRO MET LEU PHE LYS THR GLU [VAL] PRO LYS SER CYS SER ***
CTTAAACACACTTCAACCCATGTTGTTCAAGACAGAGGTTCCAAAAAGCTGCAGTTAATC
GAATTTGTGTGAAGTTGGGTACAACAAGTTCTGTCTCCAAGGTTTTTCGACGTCAATTAG
          1690      1700      1710      1720      1730      1740

ATAATTAACCGCAATATGCATTAACCTATCTATAATACAAGTATATGATAAGTAATCAGC
TATTAATTGGCGTTATACGTAATTGGATAGATATTATGTTCATATACTATTCATTAGTCG
          1750      1760      1770      1780      1790      1800

AATCAGACAATAGACAAAAGGGAAATATAAAAA
TTAGTCTGTTATCTGTTTTCCCTTTATATTTTT
          1810      1820      1830
```

NUCLEOTIDE SEQUENCE OF THE PIV-3 HN GENE. THE cDNA SEQUENCE IS SHOWN IN THE PLUS (mRNA) STRAND SENSE IN THE 5' TO 3' DIRECTION. THE TRANSMEMBRANE (TM) ANCHOR DOMAIN IS UNDERLINED. AMINO ACIDS DIFFERING FROM THE PUBLISHED PRIMARY SEQUENCE OF THE PROTEIN ENCODED BY THE PIV-3 HN GENE ARE BOXED.

FIG.3E.

RESTRICTION MAP OF THE PIV-3 HN GENE

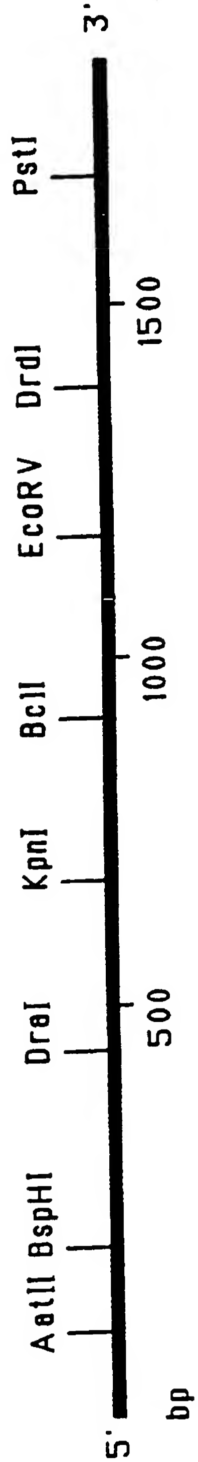

FIG.4.

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FIG.5A.

NUCLEOTIDE SEQUENCE OF THE RSV F GENE.

```
                              SP
5' MET GLU LEU PRO ILE LEU LYS ALA ASN ALA ILE THR THR ILE LEU ALA ALA VAL THR PHE
   A T G G A G T T G C C A A T C C T C A A A G C A A A T G C A A T T A C C A C A A T C C T C G C T G C A G T C A C A T T T
   T A C C T C A A C G G T T A G G A G T T T C G T T T A C G T T A A T G G T G T T A G G A G C G A C G T C A G T G T A A A
                     10                  20                  30                  40                  50                  60

   CYS PHE ALA SER SER GLN ASN ILE THR GLU GLU PHE TYR GLN SER THR CYS SER ALA VAL
   T G C T T T G C T T C T A G T C A A A A C A T C A C T G A A G A A T T T T A T C A A T C A A C A T G C A G T G C A G T T
   A C G A A A C G A A G A T C A G T T T T G T A G T G A C T T C T T A A A A T A G T T A G T T G T A C G T C A C G T C A A
                     70                  80                  90                 100                 110                 120

   SER LYS GLY TYR LEU SER ALA LEU ARG THR GLY TRP TYR THR SER VAL ILE THR ILE GLU
   A G C A A A G G C T A T C T T A G T G C T C T A A G A A C T G G T T G G T A T A C T A G T G T T A T A A C T A T A G A A
   T C G T T T C C G A T A G A A T C A C G A G A T T C T T G A C C A A C C A T A T G A T C A C A A T A T T G A T A T C T T
                    130                 140                 150                 160                 170                 180

   LEU SER ASN ILE LYS GLU ASN LYS CYS ASN GLY THR ASP ALA LYS VAL LYS LEU MET LYS
   T T A A G T A A T A T C A A G G A A A A T A A G T G T A A T G G A A C A G A T G C T A A G G T A A A A T T G A T G A A A
   A A T T C A T T A T A G T T C C T T T T A T T C A C A T T A C C T T G T C T A C G A T T C C A T T T T A A C T A C T T T
                    190                 200                 210                 220                 230                 240

   GLN GLU LEU ASP LYS TYR LYS ASN ALA VAL THR GLU LEU GLN LEU LEU MET GLN SER THR
   C A A G A A T T A G A T A A A T A T A A A A A T G C T G T A A C A G A A T T G C A G T T G C T C A T G C A A A G C A C A
   G T T C T T A A T C T A T T T A T A T T T T T A C G A C A T T G T C T T A A C G T C A A C G A G T A C G T T T C G T G T
                    250                 260                 270                 280                 290                 300

   PRO ALA ALA ASN ASN ARG ALA ARG ARG GLU LEU PRO ARG PHE MET ASN TYR THR LEU ASN
   C C A G C A G C A A A C A A T C G A G C C A G A A G A G A A C T A C C A A G G T T T A T G A A T T A T A C A C T C A A C
   G G T C G T C G T T T G T T A G C T C G G T C T T C T C T T G A T G G T T C C A A A T A C T T A A T A T G T G A G T T G
                    310                 320                 330                 340                 350                 360
```

```
                                                                   F2-F1 CLEAVAGE SITE
ASN THR LYS LYS THR ASN VAL THR LEU SER LYS LYS ARG LYS ARG ARG↓PHE LEU GLY PHE
AATACCAAAAAAACCAATGTAACATTAAGCAAGAAAAGGAAAAGAAGATTTCTTGGTTTT
TTATGGTTTTTTTGGTTACATTGTAATTCGTTCTTTTCCTTTTCTTCTAAAGAACCAAAA
         370       380       390       400       410       420

LEU LEU GLY VAL GLY SER ALA ILE ALA SER GLY ILE ALA VAL SER LYS VAL LEU HIS LEU
TTGTTAGGTGTTGGATCTGCAATCGCCAGTGGCATTGCTGTATCTAAGGTCCTGCACTTA
AACAATCCACAACCTAGACGTTAGCGGTCACCGTAACGACATAGATTCCAGGACGTGAAT
         430       440       450       460       470       480

GLU GLY GLU VAL ASN LYS ILE LYS SER ALA LEU LEU SER THR ASN LYS ALA VAL VAL SER
GAAGGAGAAGTGAACAAGATCAAAAGTGCTCTACTATCCACAAACAAGGCCGTAGTCAGT
CTTCCTCTTCACTTGTTCTAGTTTTCACGAGATGATAGGTGTTTGTTCCGGCATCAGTCA
         490       500       510       520       530       540

LEU SER ASN GLY VAL SER VAL LEU THR SER LYS VAL LEU ASP LEU LYS ASN TYR ILE ASP
TTATCAAATGGAGTTAGTGTCTTAACCAGCAAAGTGTTAGACCTCAAAAACTATATAGAT
AATAGTTTACCTCAATCACAGAATTGGTCGTTTCACAATCTGGAGTTTTTGATATATCTA
         550       560       570       580       590       600

LYS GLN LEU LEU PRO ILE VAL ASN LYS ARG SER CYS ARG ILE SER ASN ILE GLU THR VAL
AAACAATTGTTACCTATTGTGAATAAGCGAAGCTGCAGAATATCAAATATAGAAACTGTG
TTTGTTAACAATGGATAACACTTATTCGCTTCGACGTCTTATAGTTTATATCTTTGACAC
         610       620       630       640       650       660

ILE GLU PHE GLN HIS LYS ASN ASN ARG LEU LEU GLU ILE THR ARG GLU PHE SER VAL ASN
ATAGAGTTCCAACACAAGAACAACAGACTACTAGAGATTACCAGGGAATTTAGTGTTAAT
TATCTCAAGGTTGTGTTCTTGTTGTCTGATGATCTCTAATGGTCCCTTAAATCACAATTA
         670       680       690       700       710       720

ALA GLY VAL THR THR PRO VAL SER THR TYR MET LEU THR ASN SER GLU LEU LEU SER LEU
GCAGGTGTAACTACACCTGTAAGCACTTACATGTTAACTAATAGTGAATTATTGTCATTA
CGTCCACATTGATGTGGACATTCGTGAATGTACAATTGATTATCACTTAATAACAGTAAT
         730       740       750       760       770       780
```

FIG.5B.

```
ILE ASN ASP MET PRO ILE THR ASN ASP GLN LYS LYS LEU MET SER ASN ASN VAL GLN ILE
ATCAATGATATGCCTATAACAAATGATCAGAAAAAGTTAATGTCCAACAATGTTCAAATA
TAGTTACTATACGGATATTGTTTACTAGTCTTTTTCAATTACAGGTTGTTACAAGTTTAT
     790      800      810      820      830      840

VAL ARG GLN GLN SER TYR SER ILE MET SER ILE ILE LYS GLU GLU VAL LEU ALA TYR VAL
GTTAGACAGCAAAGTTACTCTATCATGTCCATAATAAAAGAGGAAGTCTTAGCATATGTA
CAATCTGTCGTTTCAATGAGATAGTACAGGTATTATTTTCTCCTTCAGAATCGTATACAT
     850      860      870      880      890      900

VAL GLN LEU PRO LEU TYR GLY VAL ILE ASP THR PRO CYS TRP LYS LEU HIS THR SER PRO
GTACAATTACCACTATATGGTGTGATAGATACACCTTGTTGGAAATTACACACATCCCCT
CATGTTAATGGTGATATACCACACTATCTATGTGGAACAACCTTTAATGTGTGTAGGGGA
     910      920      930      940      950      960

LEU CYS THR THR ASN THR LYS GLU GLY SER ASN ILE CYS LEU THR ARG THR ASP ARG GLY
CTATGTACAACCAACACAAAAGAAGGGTCAAACATCTGTTTAACAAGAACTGACAGAGGA
GATACATGTTGGTTGTGTTTTCTTCCCAGTTTGTAGACAAATTGTTCTTGACTGTCTCCT
     970      980      990      1000     1010     1020

TRP TYR CYS ASP ASN ALA GLY SER VAL SER PHE PHE PRO GLN ALA GLU THR CYS LYS VAL
TGGTACTGTGACAATGCAGGATCAGTATCTTTCTTCCCACAAGCTGAAACATGTAAAGTT
ACCATGACACTGTTACGTCCTAGTCATAGAAAGAAGGGTGTTCGACTTTGTACATTTCAA
     1030     1040     1050     1060     1070     1080

GLN SER ASN ARG VAL PHE CYS ASP THR MET ASN SER LEU THR LEU PRO SER GLU VAL ASN
CAATCGAATCGAGTATTTTGTGACACAATGAACAGTTTAACATTACCAAGTGAAGTAAAT
GTTAGCTTAGCTCATAAAACACTGTGTTACTTGTCAAATTGTAATGGTTCACTTCATTTA
     1090     1100     1110     1120     1130     1140

LEU CYS ASN VAL ASP ILE PHE ASN PRO LYS TYR ASP CYS LYS ILE MET THR SER LYS THR
CTCTGCAATGTTGACATATTCAATCCCAAATATGATTGTAAAATTATGACTTCAAAAACA
GAGACGTTACAACTGTATAAGTTAGGGTTTATACTAACATTTTAATACTGAAGTTTTTGT
     1150     1160     1170     1180     1190     1200
```

FIG.5C.

```
ASP VAL SER SER SER VAL ILE THR SER LEU GLY ALA ILE VAL SER CYS TYR GLY LYS THR
GATGTAAGCAGCTCCGTTATCACATCTCTAGGAGCCATTGTGTCATGCTATGGCAAAACT
CTACATTCGTCGAGGCAATAGTGTAGAGATCCTCGGTAACACAGTACGATACCGTTTTGA
         1210      1220      1230      1240      1250      1260

LYS CYS THR ALA SER ASN LYS ASN ARG GLY ILE ILE LYS THR PHE SER ASN GLY CYS ASP
AAATGTACAGCATCCAATAAAAATCGTGGAATCATAAAGACATTTTCTAACGGGTGTGAT
TTTACATGTCGTAGGTTATTTTTAGCACCTTAGTATTTCTGTAAAAGATTGCCCACACTA
         1270      1280      1290      1300      1310      1320

TYR VAL SER ASN LYS GLY VAL ASP THR VAL SER VAL GLY ASN THR LEU TYR TYR VAL ASN
TATGTATCAAATAAAGGGGTGGACACTGTGTCTGTAGGTAACACATTATATTATGTAAAT
ATACATAGTTTATTTCCCCACCTGTGACACAGACATCCATTGTGTAATATAATACATTTA
         1330      1340      1350      1360      1370      1380

LYS GLN GLU GLY LYS SER LEU TYR VAL LYS GLY GLU PRO ILE ILE ASN PHE TYR ASP PRO
AAGCAAGAAGGCAAAAGTCTCTATGTAAAAGGTGAACCAATAATAAATTTCTATGACCCA
TTCGTTCTTCCGTTTTCAGAGATACATTTTCCACTTGGTTATTATTTAAAGATACTGGGT
         1390      1400      1410      1420      1430      1440

LEU VAL PHE PRO SER ASP GLU PHE ASP ALA SER ILE SER GLN VAL ASN GLU LYS ILE ASN
TTAGTATTCCCCTCTGATGAATTTGATGCATCAATATCTCAAGTCAACGAGAAGATTAAC
AATCATAAGGGGAGACTACTTAAACTACGTAGTTATAGAGTTCAGTTGCTCTTCTAATTG
         1450      1460      1470      1480      1490      1500

GLN SER LEU ALA PHE ILE ARG LYS SER ASP GLU LEU LEU HIS ASN VAL ASN ALA GLY LYS
CAGAGTTTAGCATTTATTCGTAAATCCGATGAATTATTACATAATGTAAATGCTGGTAAA
GTCTCAAATCGTAAATAAGCATTTAGGCTACTTAATAATGTATTACATTTACGACCATTT
         1510      1520      1530      1540      1550      1560
                  <------------------- TM -------------------

SER THR THR ASN ILE MET ILE THR THR ILE ILE ILE GLU ILE ILE VAL ILE LEU LEU SER
TCAACCACAAATATCATGATAACTACTATAATTATAGAGATTATAGTAATATTGTTATCA
AGTTGGTGTTTATAGTACTATTGATGATATTAATATCTCTAATATCATTATAACAATAGT
         1570      1580      1590      1600      1610      1620
```

FIG.5D.

```
LEU ILE ALA VAL GLY LEU LEU LEU TYR CYS LYS ALA ARG SER THR PRO VAL THR LEU SER
TTAATTGCTGTTGGACTGCTCCTATACTGTAAGGCCAGAAGCACACCAGTCACACTAAGC
AATTAACGACAACCTGACGAGGATATGACATTCCGGTCTTCGTGTGGTCAGTGTGATTCG
      1630      1640      1650      1660      1670      1680

LYS, ASP GLN LEU SER GLY ILE ASN ASN ILE ALA PHE SER ASN
AAGGATCAACTGAGTGGTATAAATAATATTGCATTTAGTAACTGAATAAAAATAGCACCT
TTCCTAGTTGACTCACCATATTTATTATAACGTAAATCATTGACTTATTTTTATCGTGGA
      1690      1700      1710      1720      1730      1740

AATCATGTTCTTACAATGGTTTACTATCTGCTCATAGACAACCCATCTATCATTGGATTT
TTAGTACAAGAATGTTACCAAATGATAGACGAGTATCTGTTGGGTAGATAGTAACCTAAA
      1750      1760      1770      1780      1790      1800

TCTTAAAATCTGAACTTCATCGAAACTCTTATCTATAAACCATCTCACTTACACTATTTA
AGAATTTTAGACTTGAAGTAGCTTTGAGAATAGATATTTGGTAGAGTGAATGTGATAAAT
      1810      1820      1830      1840      1850      1860

AGTAGATTCCTAGTTTATAGTTATAT 3'
TCATCTAAGGATCAAATATCAATATA
      1870      1880
```

NUCLEOTIDE SEQUENCE OF THE RSV F GENE.THE cDNA SEQUENCE IS SHOWN IN THE PLUS (mRNA) STRAND SENSE IN THE 5' TO 3' DIRECTION.THE SIGNAL PEPTIDE (SP) AND THE TRANSMEMBRANE (TM) ANCHOR DOMAIN ARE UNDERLINED.THE PREDICTED F2-F1 CLEAVAGE SITE IS INDICATED BY THE ARROW (↓).AMINO ACIDS DIFFERING FROM THE PUBLISHED PRIMARY SEQUENCE OF THE PROTEIN ENCODED BY THE RSV F GENE ARE BOXED.

FIG.5E.

RESTRICTION MAP OF THE RSV F GENE

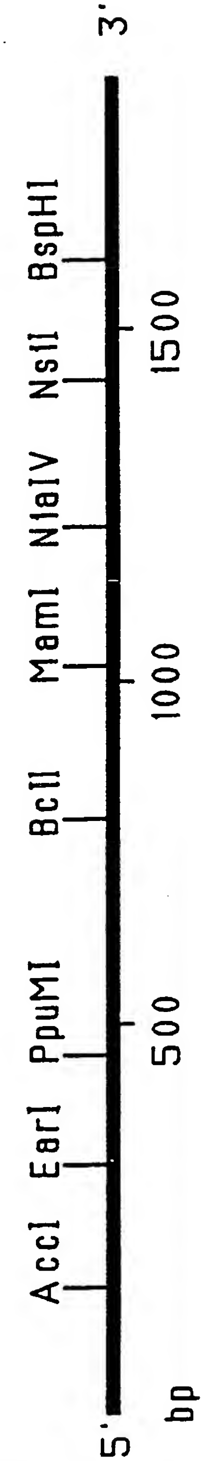


FIG.6.

FIG.7A.

NUCLEOTIDE SEQUENCE OF THE RSV G GENE

```
              MET   SER   LYS   ASN   LYS   ASP   GLN   ARG
T G C A A A C A T G T C C A A A A A C A A G G A C C A A C G
A C G T T T G T A C A G G T T T T T G T T C C T G G T T G C
                10                  20                  30

  THR   ALA   LYS   THR   LEU   GLU   LYS   THR   TRP   ASP
C A C C G C T A A G A C A C T A G A A A A G A C C T G G G A
G T G G C G A T T C T G T G A T C T T T T C T G G A C C C T
                40                  50                  60

  THR   LEU   ASN   HIS   LEU   LEU   PHE   ILE   SER   SER
C A C T C T C A A T C A T T T A T T A T T C A T A T C A T C
G T G A G A G T T A G T A A A T A A T A A G T A T A G T A G
                70                  80                  90
                                                    <----

  GLY   LEU   TYR   LYS   LEU   ASN   LEU   LYS   SER   VAL
G G G C T T A T A T A A G T T A A A T C T T A A A T C T G T
C C C G A A T A T A T T C A A T T T A G A A T T T A G A C A
               100                 110                 120
------------------------ TM ---------------------------------
  ALA   GLN   ILE   THR   LEU   SER   ILE   LEU   ALA   MET
A G C A C A A A T C A C A T T A T C C A T T C T G G C A A T
T C G T G T T T A G T G T A A T A G G T A A G A C C G T T A
               130                 140                 150
-------------------------------------------------------------
  ILE   ILE   SER   THR   SER   LEU   ILE   ILE   THR   ALA
G A T A A T C T C A A C T T C A C T T A T A A T T A C A G C
C T A T T A G A G T T G A A G T G A A T A T T A A T G T C G
               160                 170                 180
-------------------------------------------------->
  ILE   ILE   PHE   ILE   ALA   SER   ALA   ASN   HIS   LYS
C A T C A T A T T C A T A G C C T C G G C A A A C C A C A A
G T A G T A T A A G T A T C G G A G C C G T T T G G T G T T
               190                 200                 210

  VAL   THR   LEU   THR   THR   ALA   ILE   ILE   GLN   ASP
A G T C A C A C T A A C A A C T G C A A T C A T A C A A G A
T C A G T G T G A T T G T T G A C G T T A G T A T G T T C T
               220                 230                 240

  ALA   THR   SER   GLN   ILE   LYS   ASN   THR   THR   PRO
T G C A A C A A G C C A G A T C A A G A A C A C A A C C C C
A C G T T G T T C G G T C T A G T T C T T G T G T T G G G G
               250                 260                 270

  THR   TYR   LEU   THR   GLN   ASP   PRO   GLN   LEU   GLY
A A C A T A C C T C A C T C A G G A T C C T C A G C T T G G
T T G T A T G G A G T G A G T C C T A G G A G T C G A A C C
               280                 290                 300
```

FIG.7B.

ILE SER $\boxed{\text{PHE}}$ SER ASN $\boxed{\text{LEU}}$ SER GLU ILE THR

A A T C A G C T T C T C C A A T C T G T C T G A A A T T A C

T T A G T C G A A G A G G T T A G A C A G A C T T T A A T G

310 320 330

SER GLN $\boxed{\text{THR}}$ THR THR ILE LEU ALA SER THR

A T C A C A A A C C A C C A C C A T A C T A G C T T C A A C

T A G T G T T T G G T G G T G G T A T G A T C G A A G T T G

340 350 360

THR PRO GLY VAL LYS SER $\boxed{\text{ASN}}$ LEU GLN $\boxed{\text{PRO}}$

A A C A C C A G G A G T C A A G T C A A A C C T G C A A C C

T T G T G G T C C T C A G T T C A G T T T G G A C G T T G G

370 380 390

THR THR VAL LYS THR LYS ASN THR THR THR

C A C A A C A G T C A A G A C T A A A A A C A C A A C A A C

G T G T T G T C A G T T C T G A T T T T T G T G T T G T T G

400 410 420

THR GLN THR GLN PRO SER LYS PRO THR THR

A A C C C A A A C A C A A C C C A G C A A G C C C A C T A C

T T G G G T T T G T G T T G G G T C G T T C G G G T G A T G

430 440 450

LYS GLN ARG GLN ASN LYS PRO PRO $\boxed{\text{ASN}}$ LYS

A A A A C A A C G C C A A A A C A A A C C A C C A A A C A A

T T T T G T T G C G G T T T T G T T T G G T G G T T T G T T

460 470 480

PRO ASN ASN ASP PHE HIS PHE GLU VAL PHE

A C C C A A T A A T G A T T T T C A C T T C G A A G T G T T

T G G G T T A T T A C T A A A A G T G A A G C T T C A C A A

490 500 510

ASN PHE VAL PRO CYS SER ILE CYS SER ASN

T A A C T T T G T A C C C T G C A G C A T A T G C A G C A A

A T T G A A A C A T G G G A C G T C G T A T A C G T C G T T

520 530 540

ASN PRO THR CYS TRP ALA ILE CYS LYS ARG

C A A T C C A A C C T G C T G G G C T A T C T G C A A A A G

G T T A G G T T G G A C G A C C C G A T A G A C G T T T T C

550 560 570

ILE PRO ASN LYS LYS PRO GLY LYS LYS THR

A A T A C C A A A C A A A A A A C C A G G A A A G A A A A C

T T A T G G T T T G T T T T T T G G T C C T T T C T T T T G

580 590 600

SUBSTITUTE SHEET

FIG.7C.

THR THR LYS PRO THR LYS LYS PRO THR PHE
C A C C A C C A A G C C T A C A A A A A A A C C A A C C T T
G T G G T G G T T C G G A T G T T T T T T T G G T T G G A A
610 620 630

LYS THR THR LYS LYS ASP $\boxed{\text{LEU}}$ LYS PRO GLN
C A A G A C A A C C A A A A A A G A T C T C A A A C C T C A
G T T C T G T T G G T T T T T T C T A G A G T T T G G A G T
640 650 660

THR THR LYS $\boxed{\text{PRO}}$ LYS GLU VAL PRO THR THR
A A C C A C T A A A C C A A A G G A A G T A C C C A C C A C
T T G G T G A T T T G G T T T C C T T C A T G G G T G G T G
670 680 690

LYS PRO THR GLU GLU PRO THR ILE ASN THR
C A A G C C C A C A G A A G A G C C A A C C A T C A A C A C
G T T C G G G T G T C T T C T C G G T T G G T A G T T G T G
700 710 720

THR LYS THR ASN ILE $\boxed{\text{THR}}$ THR THR LEU LEU
C A C C A A A A C A A A C A T C A C A A C T A C A C T G C T
G T G G T T T T G T T T G T A G T G T T G A T G T G A C G A
730 740 750

THR $\boxed{\text{ASN}}$ ASN THR THR GLY ASN PRO $\boxed{\text{LYS}}$ LEU
C A C C A A C A A C A C C A C A G G A A A T C C A A A A C T
G T G G T T G T T G T G G T G T C C T T T A G G T T T T G A
760 770 780

THR SER GLN MET GLU THR PHE HIS SER THR
C A C A A G T C A A A T G G A A A C C T T C C A C T C A A C
G T G T T C A G T T T A C C T T T G G A A G G T G A G T T G
790 800 810

SER SER GLU GLY ASN $\boxed{\text{LEU}}$ SER PRO SER GLN
C T C C T C C G A A G G C A A T C T A A G C C C T T C T C A
G A G G A G G C T T C C G T T A G A T T C G G G A A G A G T
820 830 840

VAL SER THR THR SER GLU $\boxed{\text{HIS}}$ PRO SER GLN
A G T C T C C A C A A C A T C C G A G C A C C C A T C A C A
T C A G A G G T G T T G T A G G C T C G T G G G T A G T G T
850 860 870

PRO SER SER PRO PRO ASN THR $\boxed{\text{THR}}$ ARG GLN
A C C C T C A T C T C C A C C C A A C A C A A C A C G C C A
T G G G A G T A G A G G T G G G T T G T G T T G T G C G G T
880 890 900

***
G T A G T T A T T A A A A A A A A A A A
C A T C A A T A A T T T T T T T T T T T
910 920

NUCLEOTIDE SEQUENCE OF THE RSV G GENE. THE cDNA SEQUENCE IS SHOWN IN THE PLUS (mRNA) STRAND SENSE IN THE 5' TO 3' DIRECTION. THE TRANSMEMBRANE (TM) ANCHOR DOMAIN IS UNDERLINED. AMINO ACIDS DIFFERING FROM THE PUBLISHED PRIMARY SEQUENCE OF THE PROTEIN ENCODED BY THE RSV G GENE ARE BOXED.

FIG.7D.

RESTRICTION MAP OF RSV G GENE

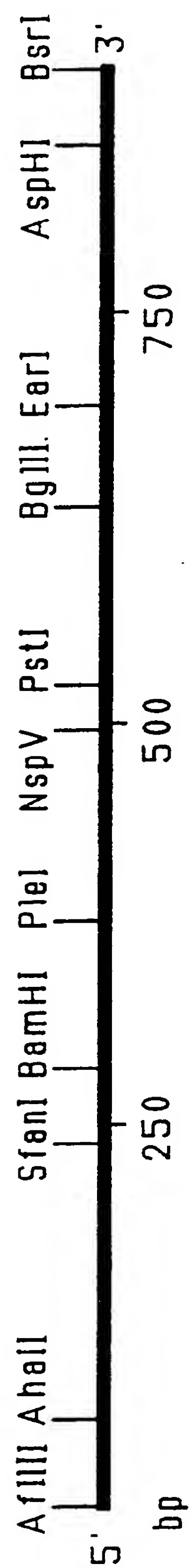


FIG.8.

Construction of a Bluescript-based expression vector containing the chimeric F_{PIV-3} -F_{RSV} gene with the 5' untranslated region of the PIV-3 F gene intact but lacking the nucleotide sequences coding for the hydrophobic anchor domains and cytoplasmic tails of both the PIV-3 and RSV F genes.

Step 1: Preparation of the plasmid containing the modified PIV-3 F gene

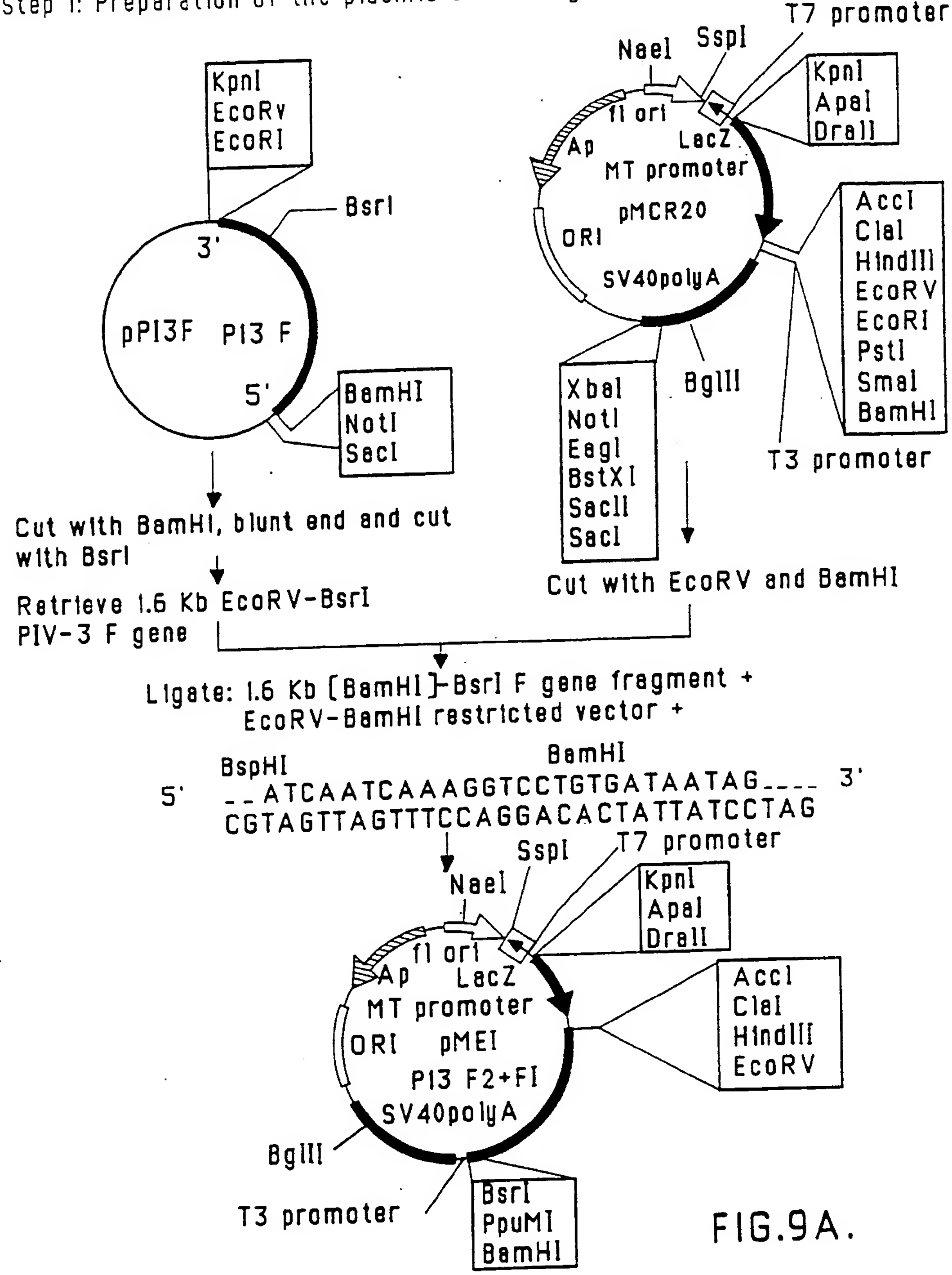
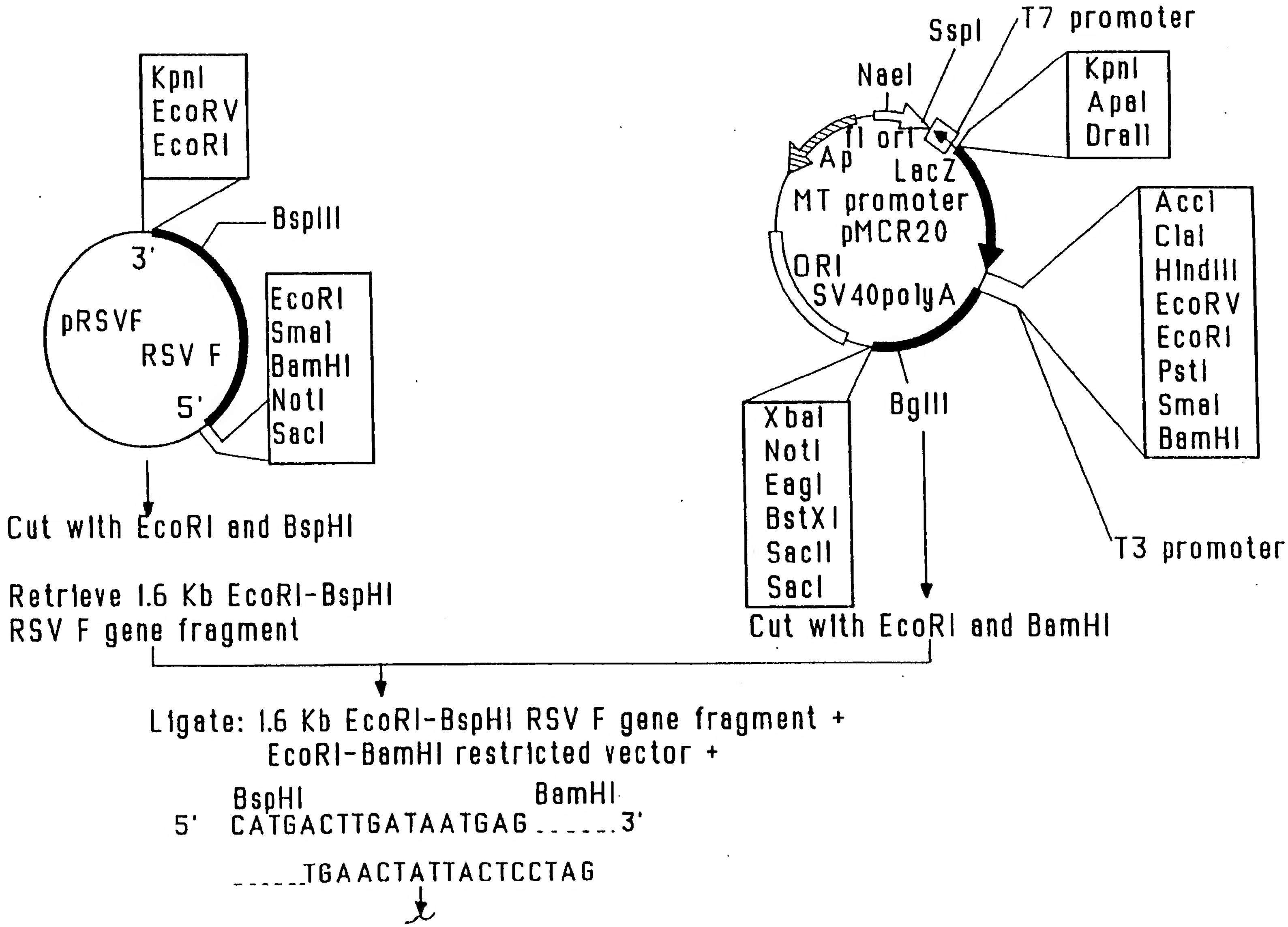
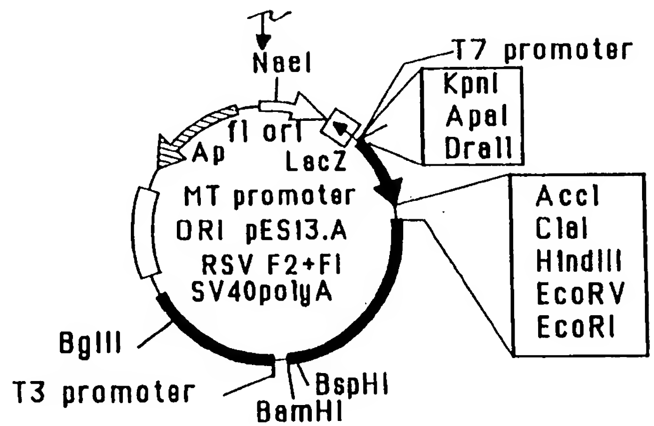


FIG.9A.

FIG.9B. Step 2: Preparation of the plasmid containing the modified RSV F gene





Cut with EcoRI and PpuMI

EcoRI PpuMI

LIGATE: 5' AATTCATGGAGTTGCTAATCCTCAAAGCAAATGCAATTAGCACAATCCTCACTGCAGTCACATTTTGTTTTGCTTCTGGTTCTAAG -------

-----GTACCTCAACGATTAGGAGTTTCGTTTACGTTAATGGTGTTAGGAGTGACGTCAGTGTAAAACAAAACGAAGACCAAGATTCCAG

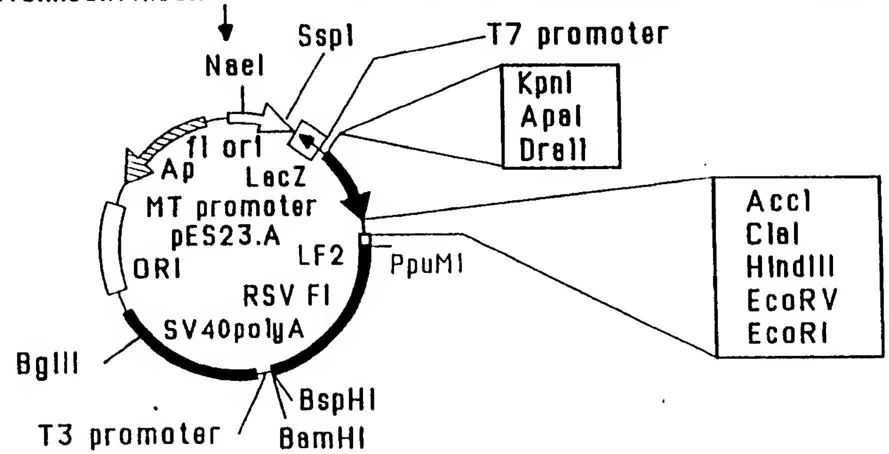


FIG.9C.

Step 3 : Preparation of the plasmid containing the chimeric F_{PIV-3} -F_{RSV} gene

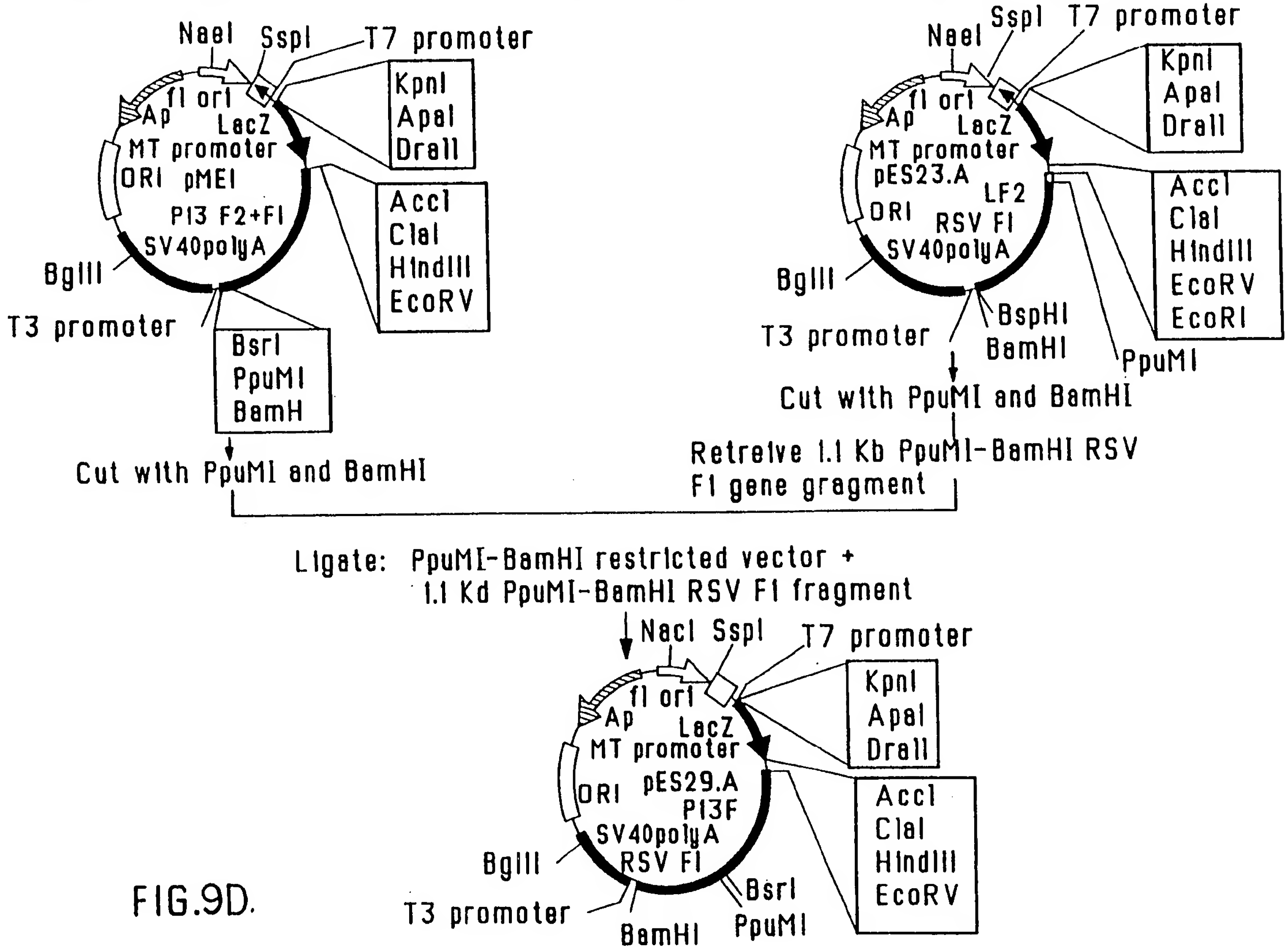


FIG.9D.

Construction of a Bluescript-based expression vector containing the PIV-3 F gene lacking the 5' untranslated sequence and transmembrane anchor and cytoplasmic tail coding regions.

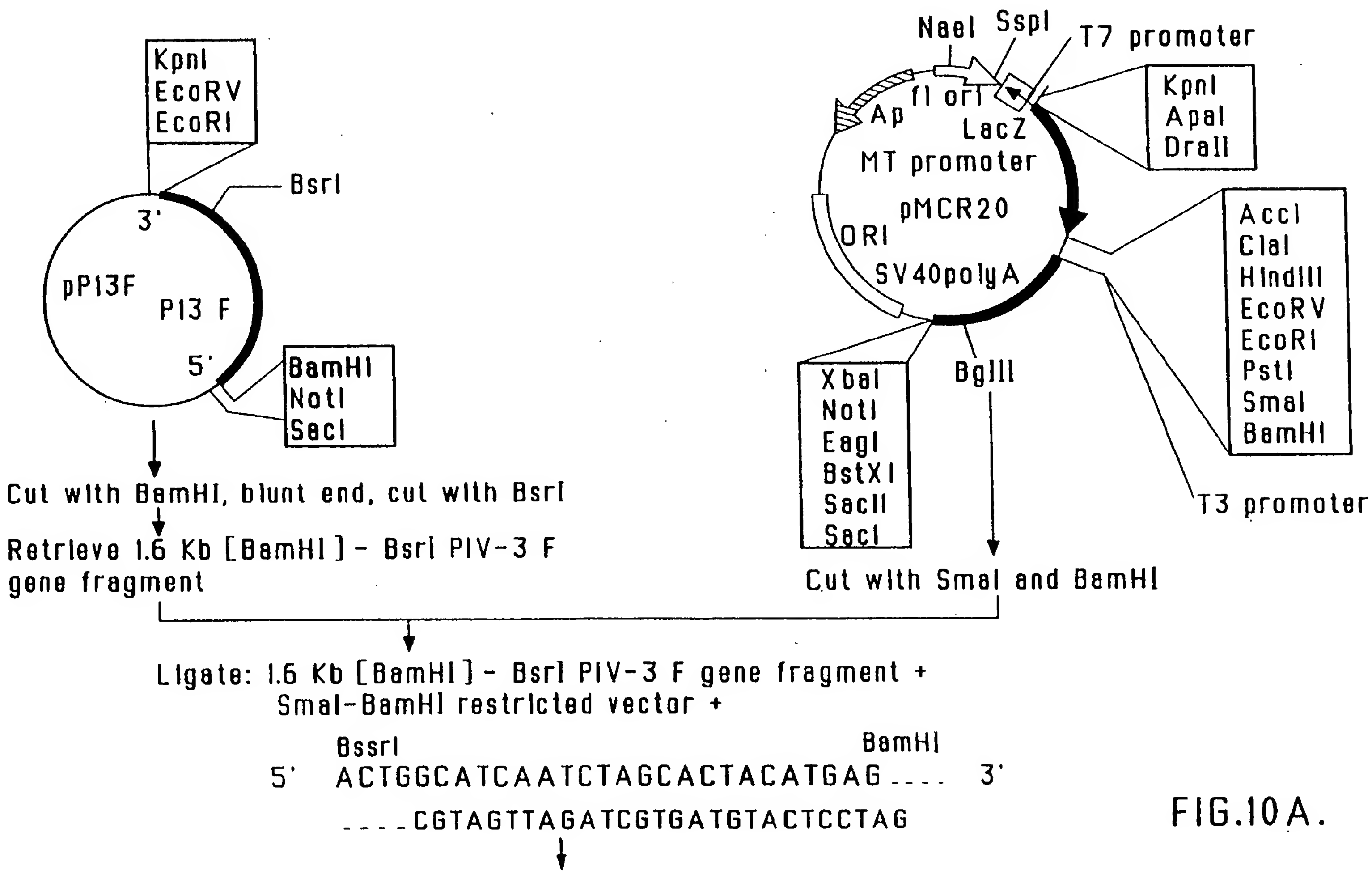
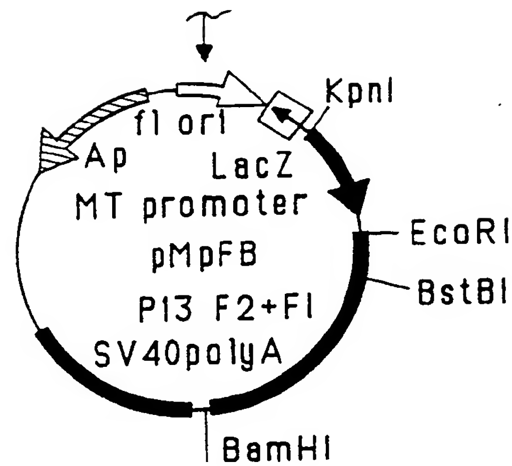


FIG.10A.

FIG.10B.



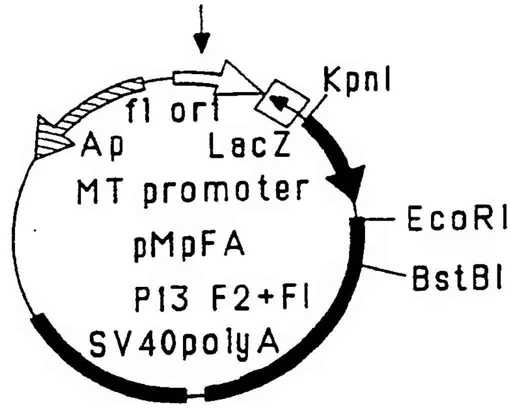
Cut with EcoRI and BstBI

Retreive: EcoRI-BstBI restricted vector

Ligate: EcoRI-BstBI restricted vector +

EcoRI PpuMI

AATTCATGCCAACTTTAATACTGCTAATTATTACAACAATGATTATGG
CATCTTCCTGCCAAATAGATATCACAAAACTACAGCAATGTAGGTGTA
TTGGTCAACAGTCCCAAAGGGATGAAGATATCACAAAACTT - - - - 3'
- - - - GTACGGTTGAAATTATGACGATTAATAATGTTGTTACTAATACC
GTAGAAGGACGGTTTATCTATAGTGTTTTGATGTCGTACATCCACATA
ACCAGTTGTCAGGGTTTCCCTACTTCTATAGTGTTTTGAAGCTT



Construction of the chimeric $F_{PIV-3}-F_{RSV}$ gene consisting of the truncated PIV-3 F gene devoid of the 5' untranslated region linked to the truncated RSV F1 gene.

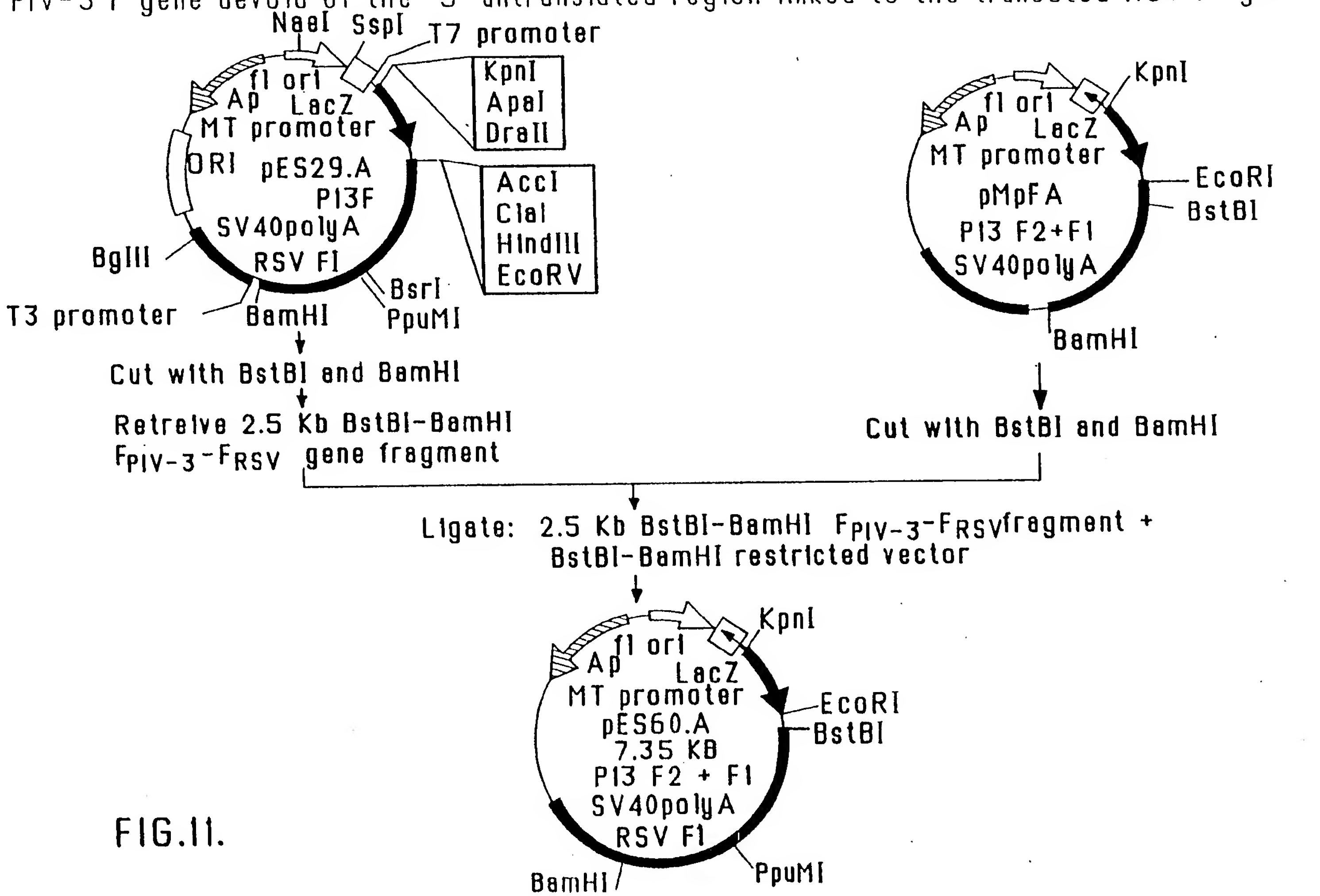
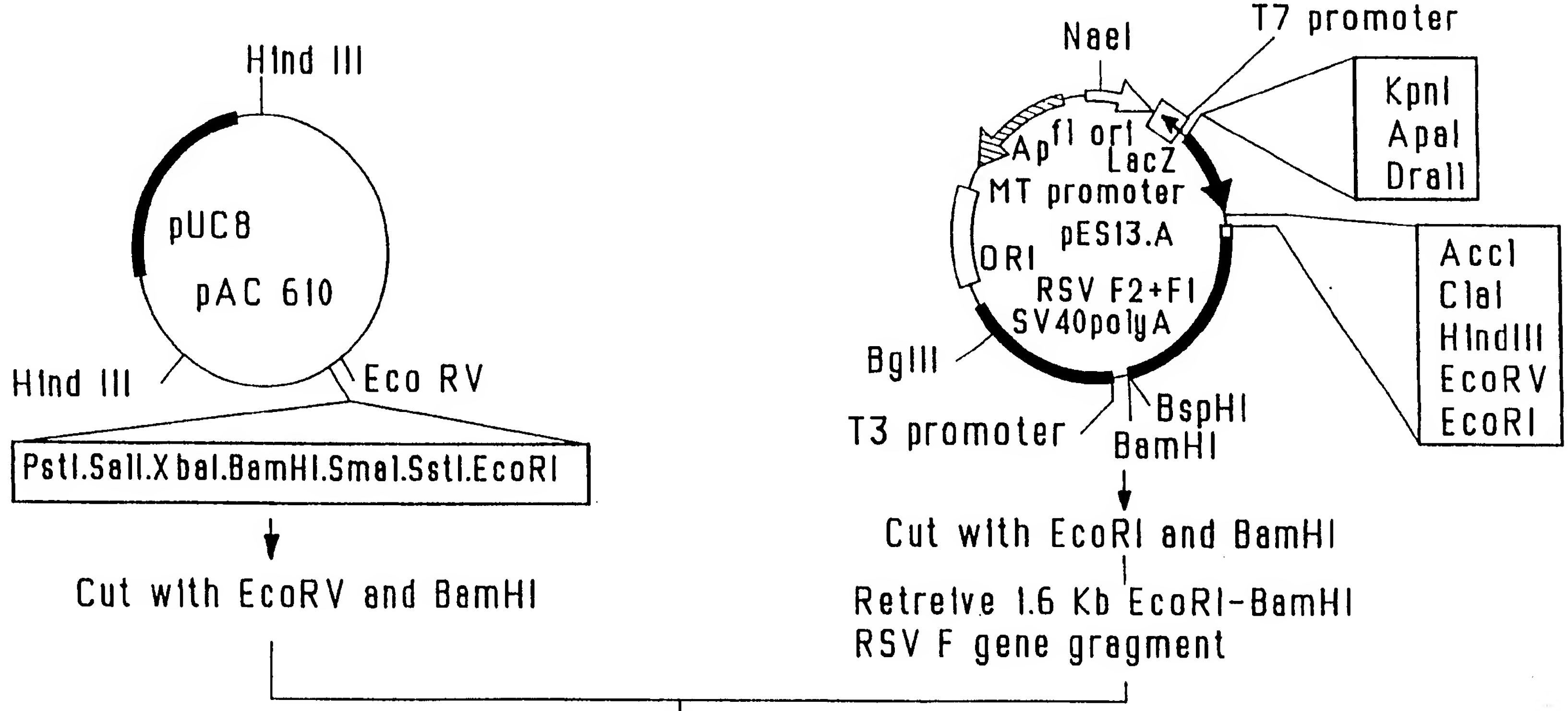


FIG.11.

FIG.12A. Construction of the modified pAc 610 baculovirus expression vector containing the chimeric $F_{PIV-3}-F_{RSV}$ gene consisting of the PIV-3 F gene lacking both the 5' untranslated sequence as well as the transmembrane and cytoplasmic tail coding regions linked to the truncated RSV F1 gene



Ligate: 1.6 Kb EcoRI-BamHI RSV F gene fragment +
EcoRV-BamHI restricted vector +

EcoRV EcoRI

5' ATCATGGAGATAATTAAAATGATAACCATCTCGCAAATAAATAAGTATTTTACTGTTTTCGTAACAGTTTTGTAATAAAAAAACCTATAAATAG 3'
TAGTACCTCTATTAATTTTACTATTGGTAGAGCGTTTATTTATTCATAAAATGACAAAAGCATTGTCAAAACATTATTTTTTTGGATATTTATCTTAA

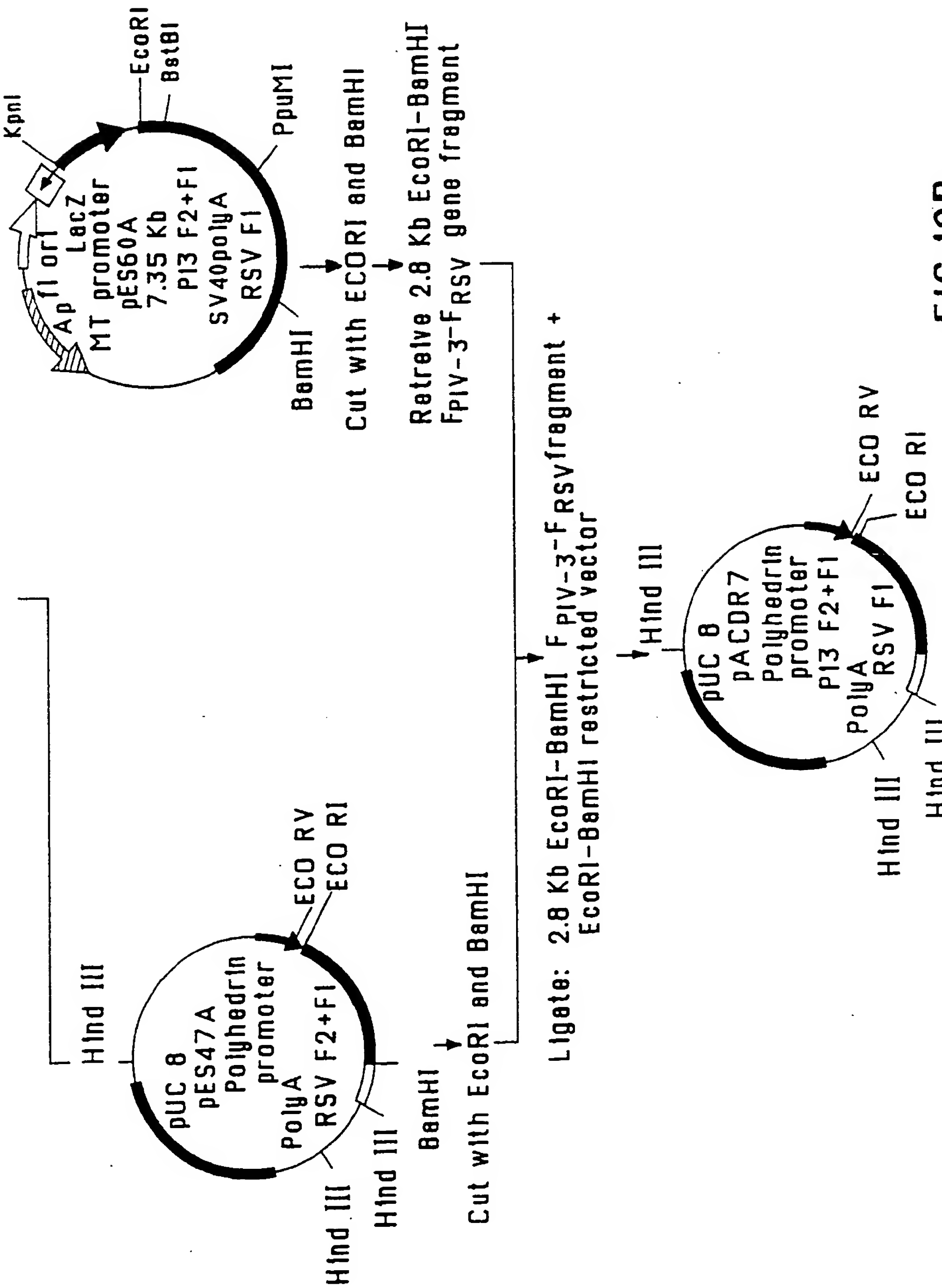

KpnI
EcoRI
BstBI
PpuMI
Ap
f1 ori
LacZ
MT promoter
pES60A
7.35 Kb
P13 F2+F1
SV40polyA
RSV F1
BamHI
Cut with ECORI and BamHI
Retreive 2.8 Kb EcoRI-BamHI
F_{PIV-3}-F_{RSV} gene fragment
Hind III
pUC 8
pES47A
Polyhedrin
promoter
PolyA
RSV F2+F1
ECO RV
ECO RI
Hind III
Hind III
BamHI
Cut with EcoRI and BamHI
Ligate: 2.8 Kb EcoRI-BamHI F_{PIV-3}-F_{RSV} fragment +
EcoRI-BamHI restricted vector
Hind III
pUC 8
pACDR7
Polyhedrin
promoter
P13 F2+F1
PolyA
RSV F1
ECO RV
ECO RI
Hind III
Hind III

FIG.12B.

FIG.13

IMMUNOBLOTS OF CELL LYSATES FROM Sf9 CELLS INFECTED WITH RECOMBINANT BACULOVIRUSES

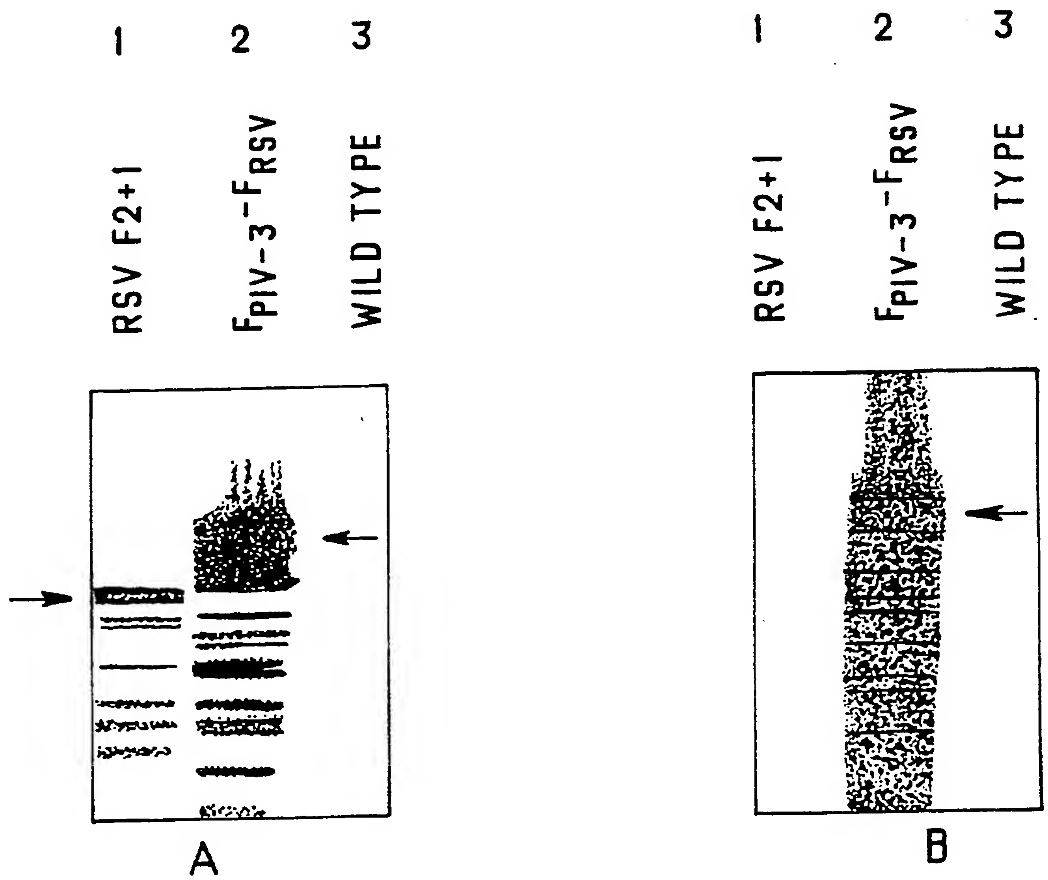


FIG 13 : Immunoblots of cell lysates from Sf9 cells infected wirth recombinant baculoviruses containing the truncated RSV F gene (Lane 1), the chimeric $F_{PIV-3}-F_{RSV}$gene (Lane 2) or infected with wild type virus (Lane 3) reacted with anti-F RSV Mab (panel A) and anti-F1 PIV-3 antiserum (panel B)

FIG.14. CONSTRUCTION OF THE BACULOVIRUS TRANSFER VECTOR pD2

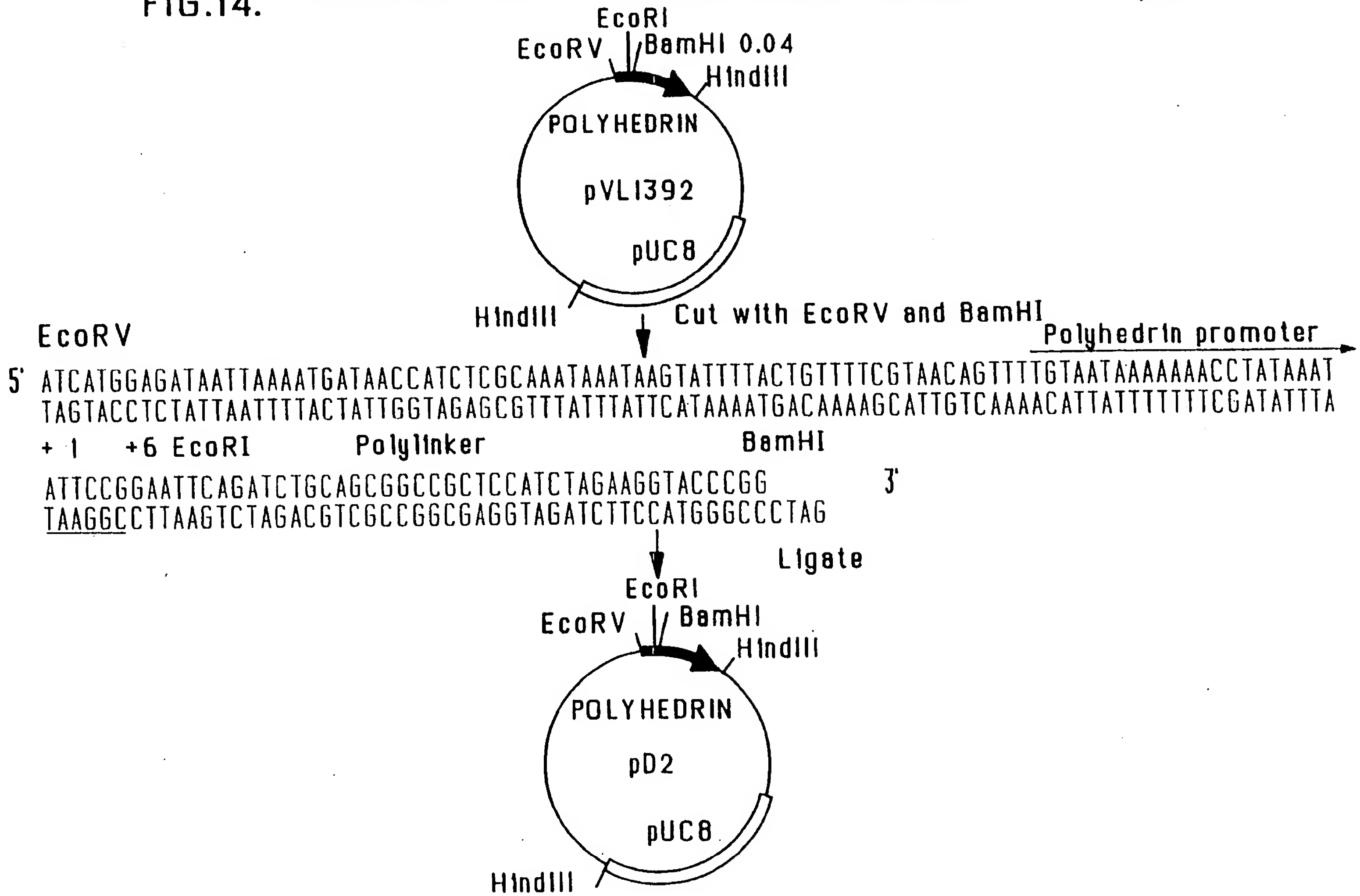


FIG.15A. CONSTRUCTION OF THE F_{RSV}-HN_{PIV3} CHIMERIC GENE

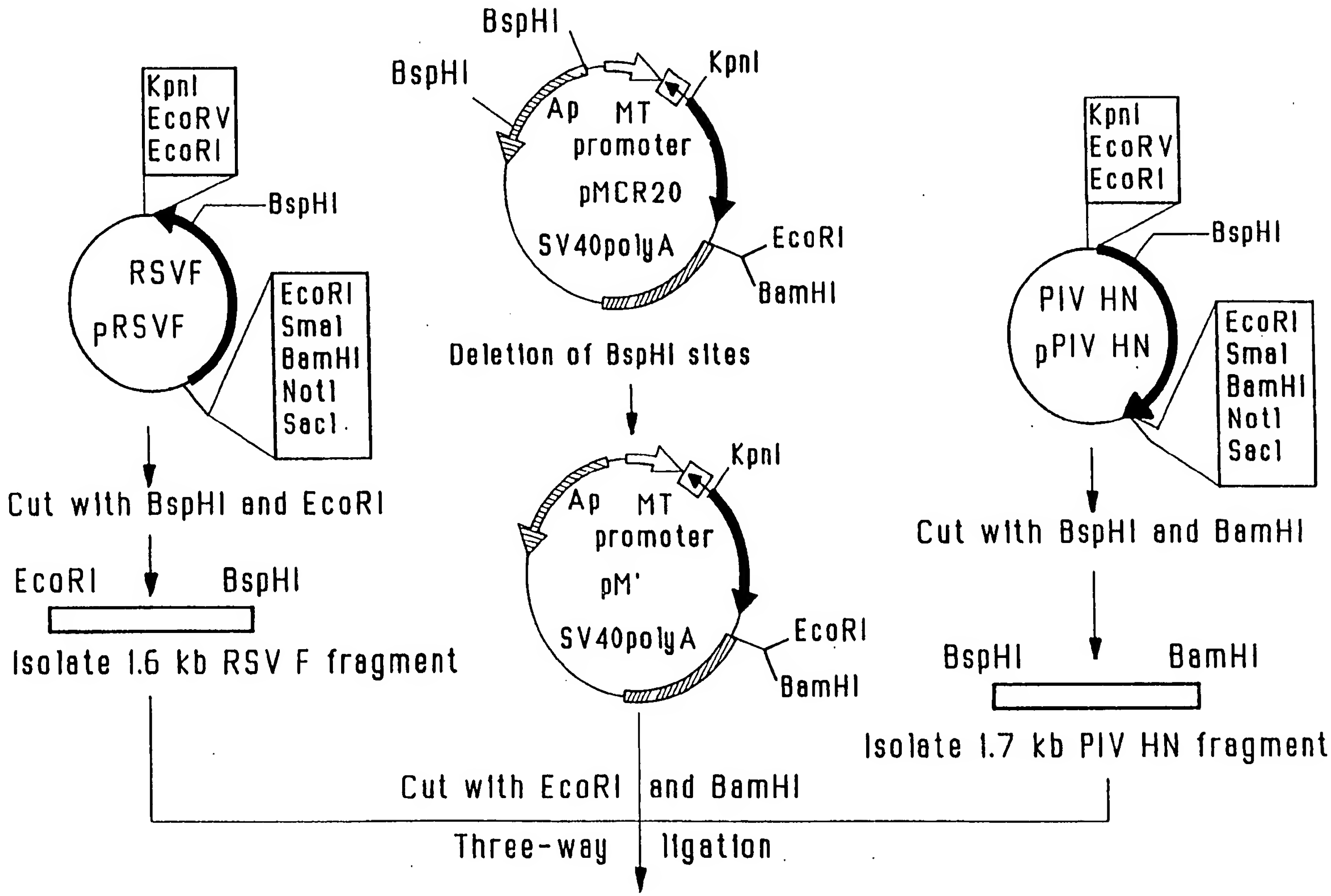


FIG.15B.

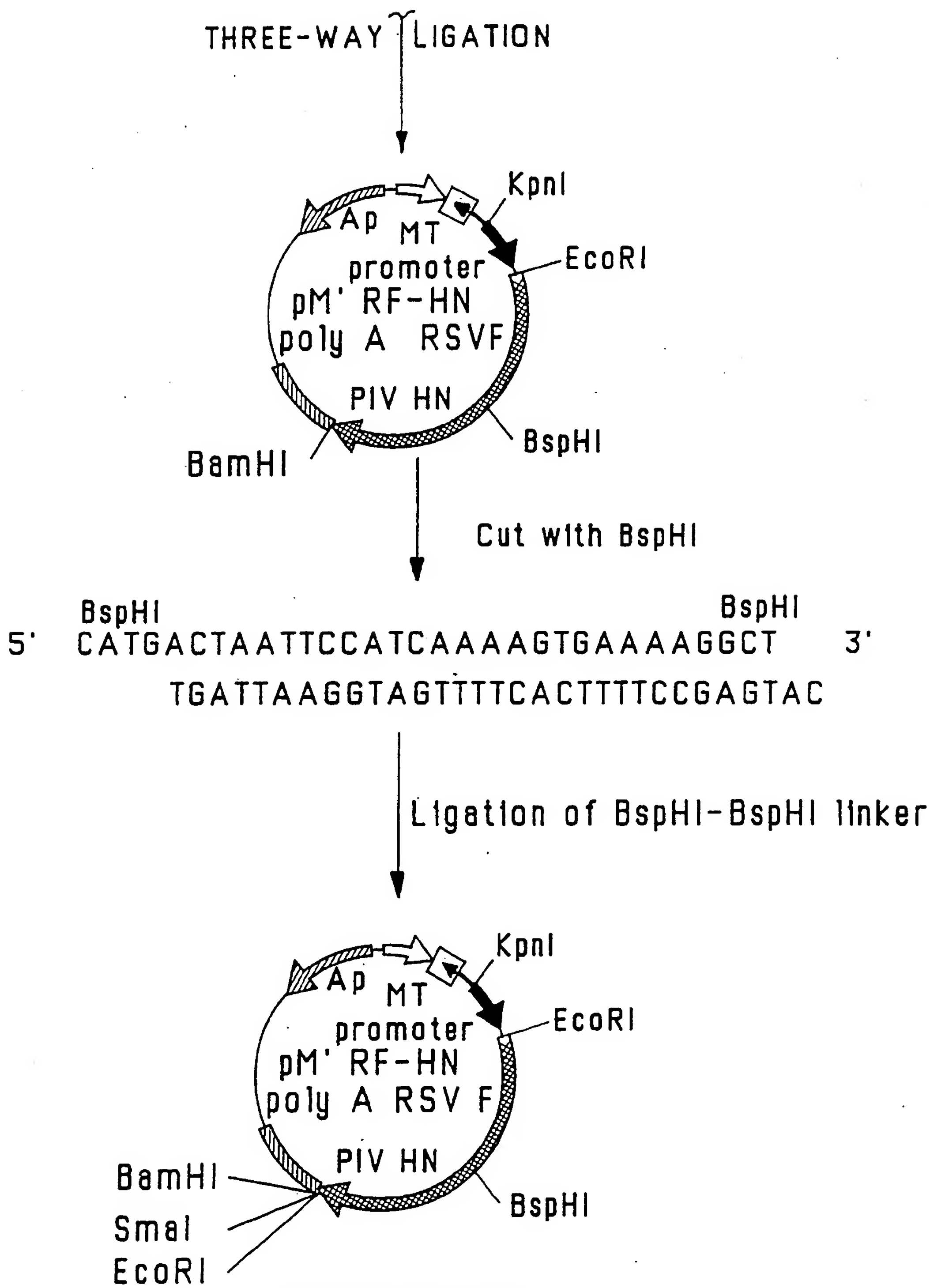

THREE-WAY LIGATION
Ap
MT
promoter
pM' RF-HN
poly A
RSVF
PIV HN
KpnI
EcoRI
BspHI
BamHI
Cut with BspHI
BspHI
BspHI
5' CATGACTAATTCCATCAAAAGTGAAAAGGCT 3'
TGATTAAGGTAGTTTTCACTTTTCCGAGTAC
Ligation of BspHI-BspHI linker
Ap
MT
promoter
pM' RF-HN
poly A RSV F
PIV HN
KpnI
EcoRI
BspHI
BamHI
SmaI
EcoRI

FIG.16

SDS POLY ACRYLAMIDE GEL AND IMMUNOBLOTS OF PURIFIED F_{RSV}-HN_{PIV-3} CHIMERIC PROTEIN

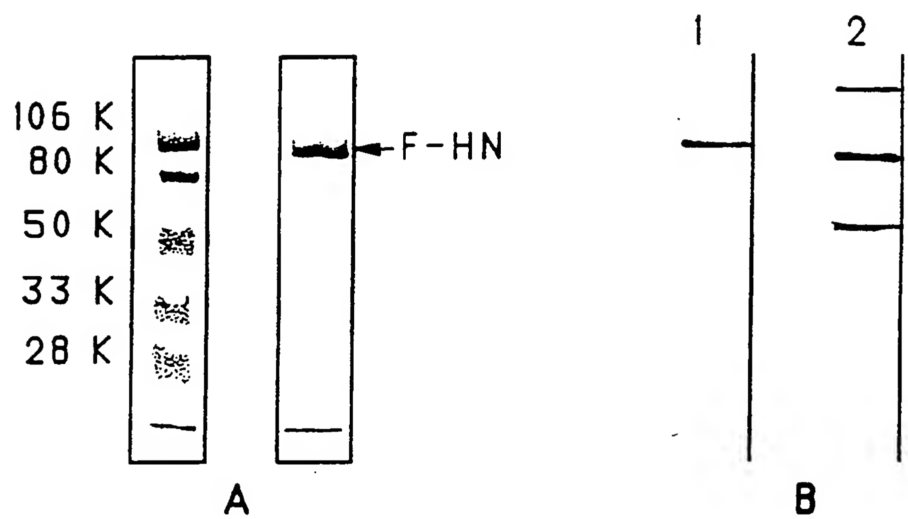


FIG 16 : A) Coomassie-stained SDS polyacrylamide gel of immunoaffinity- purified F_{RSV}-HN_{PIV-3}protein.

B) Immunoblots of F_{RSV}-HN_{PIV-3}protein reacted with an anti-F RSV Mab (lane 1) and anti-HN PIV-3 antiserum (lane 2)

FIG.17. MUTAGENESIS OF THE PIV-3 F GENE

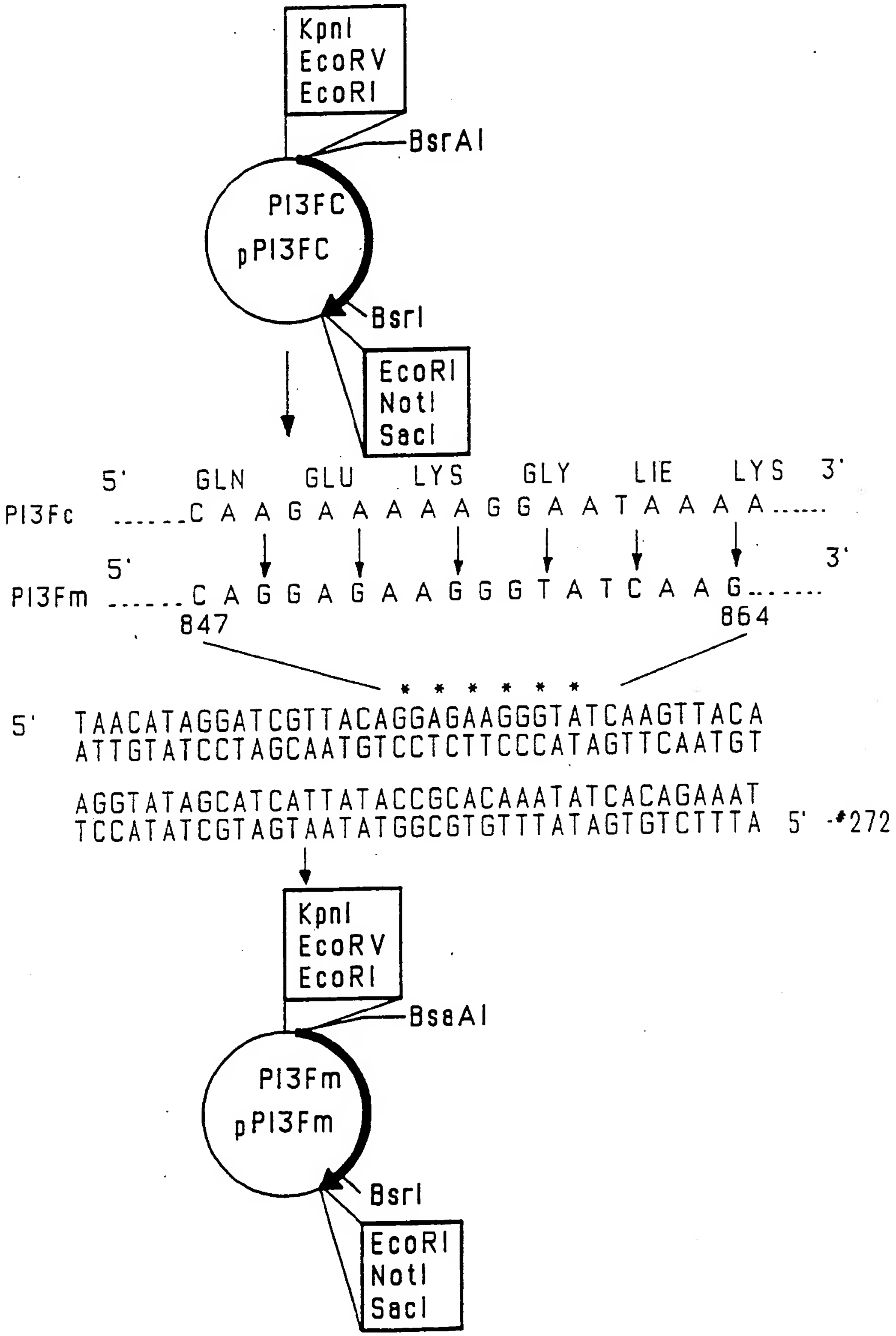
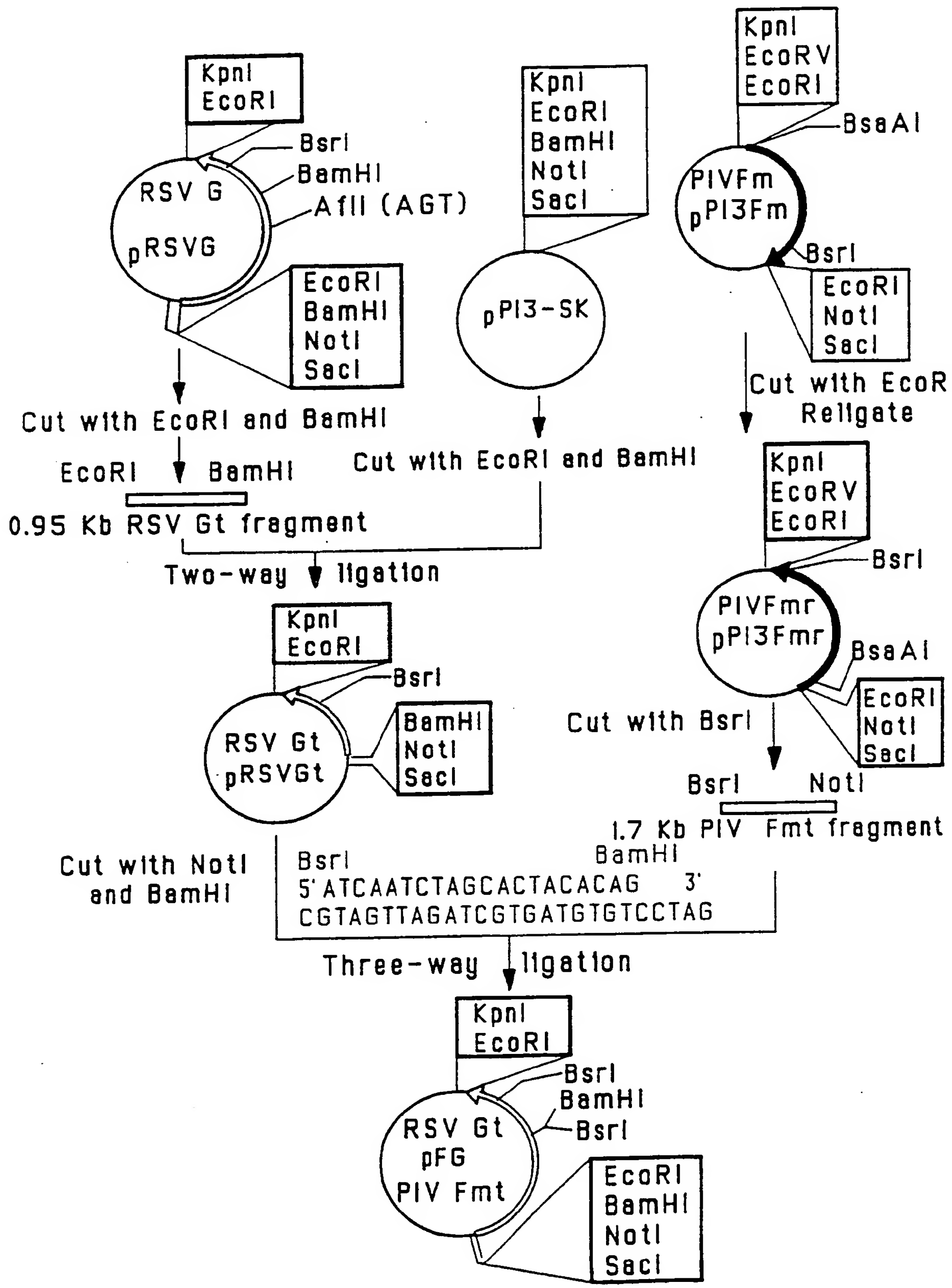


FIG.18. CONSTRUCTION OF THE F_{PIV3}-G_{RSV} CHIMERIC GENE



INTERNATIONAL SEARCH REPORT

International Applica

PCT/CA 93/00001

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/45; A61K39/155; G01N33/569

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; A61K ; G01N

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	J. VIROL. vol. 64, no. 8, 1990, pages 4007 - 4012 P. COLLINS 'O glycosylation of glycoprotein g of human respiratory syncytial virus is spaecifed within the divergen ectodomain' see the whole document ---	1-11,13, 16-26, 28,29, 32-35, 39-47,53
X	MOL. CELL. BIOL. vol. 8, no. 4, 1988, pages 1709 - 1714 S. VIJAYA ET AL. 'Transport to the cell surface of a peptide sequence attached to the truncated C terminus of an n-terminally anchored integral membrane protein' see page 1713 --- -/--	1-4,6, 16-21, 28, 32-36, 39-43, 48-54, 56-58

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

1

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
13 MAY 1993	03. 06. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	SKELLY J.M.

International Application No PCT/CA 93/00001

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	WO,A,8 910 405 (THE UPJOHN COMPANY) 2 November 1989 cited in the application see the whole document ---	1-58
Y	WO,A,8 905 823 (THE UPJOHN COMPANY) 29 June 1989 cited in the application see the whole document ---	1-58
A	J. GEN. VIROL. vol. 70, 1989, pages 2637 - 2644 R. BRIDEAU ET AL. 'Protection of cotton rats against human respiratory syncytial virus' cited in the application ---	
A	J. GEN. VIROL. vol. 70, 1989, M. WATHEN ET AL. 'Characterisation of a novel human respiratory syncytial virus chimeric FG glycoprotein' cited in the application -----	

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

CA 9300001
SA 68995

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 13/05/93
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-8910405	02-11-89	AU-B-	611784	20-06-91
		AU-A-	3197589	24-11-89
		CA-A-	1306709	25-08-92
		EP-A-	0413695	27-02-91
		US-A-	5169628	08-12-92
WO-A-8905823	29-06-89	AU-A-	2785089	19-07-89
		DE-A-	3878468	25-03-93
		EP-A,B	0396563	14-11-90
		US-A-	5194595	16-03-93

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA93/00001

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 54-56 are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.